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Biological Aspects of Cancer Research [1,2]

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The following discussion of cancer research is frankly from the biological point of view and is therefore limited in scope and application. Principles and processes of biological interest will be chiefly considered. No attempt will be made to cover in detail the vast amount of published data on experiments which contribute to definition or evaluation of the various broader subdivisions of the field. If the reader is interested in a more detailed perusal of supporting experimental evidence for general statements, he can find a partial bibliography in the writer's booklet on *Genetics, Biological Individuality, and Cancer*.[3] From this beginning he can, by covering the literature since 1954 and by tracing back from the given references, obtain some idea of the tremendous vigor of present-day research in this field and the extent and diversity of the experimental work already accomplished.

Background Research

The history of the development of the contemporary major action in the field of experimental biological research on cancer presents an interesting parallel with the last great critical battle in the war between the States.

At Gettysburg the first contacts of the opposing forces was largely a "blind" and unexpected meeting of the cavalry "antennae" of both armies. Each had little or no idea where the other was until actual contact occurred. From and around this initial center of entanglement there developed, by the assembly of more and more units of infantry and artillery, a major alignment and a fierce and final engagement of long duration and decisive significance.

In biological cancer research early experiments on animals, largely with mice, rats, and fowl, were scattered and chiefly observational. The technique of tumor transplantation was one of the most important tools and was being extensively employed by workers under the Imperial

[1] Received for publication September 23, 1957.
[2] Dr. Little was invited by the Board of Editors to express his views on general problems in cancer.
[3] Stanford University Press, 1954.

Cancer Fund in Great Britain and at the Rockefeller Institute, the Cancer Commission of Harvard University, and the Crocker Institute in this country. Continental European research was largely aimed at the discovery of infectious agents and the possible development of immunity. This point of view also activated a number of British and American investigators.

This was the stage comparable in many ways to the "cavalry" contacts with the enemy. There was similar limitation of the type of weapons which could be used and there was also the same sort of isolation and importance of individual effort or the work of small units.

It is, of course, impossible to single out all the factors disclosed by research at this stage, which later became the major elements in leading to further developments. Some, however, can be safely mentioned because their basic connection with existing and future research is clear and functional. In presenting them there is no intent to indicate relative "importance" for, until the final solution of many problems that are still unsolved or even unformulated, any rating would be unwise if not impossible.

Discovery and Isolation of Filterable Agents

From the pioneer work of Rous and his associates, the existence of a filterable agent (virus) was clearly identified as the chief etiological factor in producing sarcomas in the domestic fowl. This was an exciting and stimulating discovery. From it there developed a still active series of experiments (Claude, Duran-Reynals, Porter, and others) which gave important information on the morphology, reproduction, and modifiability of the virus and of other similar agents.

Shope described a papilloma-forming virus in the rabbit and thereby demonstrated that the mammalian cell can include a virus which actually allows an increased rate of cell division.

The staff of the Jackson Laboratory, stimulated by the recognition of a strong maternal influence in the etiology of mouse mammary adenocarcinomas (Murray, Little), published in 1933 evidence of the general existence of this phenomenon in several high-tumor strains. In the same laboratory, Bittner determined that the agent which caused this maternal influence was ordinarily transmitted in milk during nursing by the young. The agent was later shown to be present in the blood and in various body tissues of both males and females of "high-tumor" strains (Woolley, Little). Its selective distribution was later demonstrated by Hummel who found that it was absent from the placenta. The fact that it apparently remained inactive in the intact males was also highly significant and suggestive, which encourages the theory that other latent viruses may be factors in the etiology of other types of neoplasm.

The filterable nature of the mammary-tumor inciter and its immunological properties, together with some evidence of its identification under the electron microscope, have further strengthened the belief that viruses are possible etiological agents of general importance in neoplasia. The work of Gross and others on salivary-gland tumors and on leukemia in mice also

falls in with the theory that virus phenomena are a field of continuing promise for the investigator.

In pure genetic research certain similarities between viroid and genic qualities and behavior keep active the interest of research virologists and biologists in intracellular activity in relation to mitosis and molecular organization. The development of the initial and early contacts with the tumorigenic filterable agents into a full-scale, broad, and vigorous research "front" is one phase of the major "engagement" in which those investigating the cause and control of neoplasia are and will be concerned.

Genetic Theory of Tissue Transplantation

The initial objectives of tumor transplantation in animals were: (*a*) to propagate the chosen tumor in order to obtain a larger supply of tissue for continued study, (*b*) to investigate the host reaction to subcutaneous or intraperitoneal implants of auto-, homo-, and heteroplastic tissues with a view to determining the nature of the factors which determine susceptibility and nonsusceptibility. These two objectives were, of course, pursued at the same time, with the emphasis of earlier work shifting somewhat whimsically as the experimental results suggested.

Since, in most cases, little or nothing was known about the genetic nature of the animals used as hosts and since these animals differed fundamentally and invisibly from one another in their genetic characteristics, their response to inoculation was varied. Successive groups of animals inoculated gave "takes" of the tumor from 0 to 100 percent.

The British school and some Americans explained this variation as a response to changes in "virulence" in the tumor itself. Extensive papers were published dealing with such changes both in untreated animals and in those which had, before or after inoculation, received treatment with various supposedly "preventive" or "therapeutic" agents.

To add to the confusion of the unpredictability and of the irregularity of consecutive results, it was observed that temporary persistence or even growth of the tumor implant was followed, in some animals, by its regression and disappearance even in "untreated" animals. A quantitative phase was thus added to the already obscure problem.

This was the situation in the first decade of the Twentieth Century when the work of Tyzzer began to point the way to an opportunity for progress toward a more definite and satisfactory level of experimentation. This was the phase of bringing up heavier units after the "cavalry" contact.

Tyzzer had access to a strain of Japanese waltzing mice which had been used by Yerkes in extensive behavior studies, and which were being produced for research purposes by a Mrs. Lambert in a town outside of Boston. The Japanese mice were probably descended from the Asiatic mouse *Mus bactrianus* and were undoubtedly considerably inbred having been derived from an originally very restricted nucleus of animals. They were small and delicate, and later experience with their susceptibility to

infections suggests that Mrs. Lambert possessed an unusual amount of skill and patience to produce them in the numbers that she did.

These mice developed "spontaneously" a number of mammary adenocarcinomas and an occasional fibrosarcoma. Tyzzer found that such tumors, on transplantation to other mice of the Lambert strain, grew in practically 100 percent of the inoculated animals. The same tumors failed to grow in all mice of other strains. This presented Tyzzer with an opportunity to study the reaction of first- and second-generation hybrids between the 100 percent susceptible and the nonsusceptible strains.

The crosses were made and a mammary adenocarcinoma JWA was used for inoculation. *All* the first generation hybrids grew the tumor. Surprisingly *none* of the 70-odd second-generation hybrids grew it. At this point the writer took over the experiment and repeated the crosses. As before, all F_1 hybrids were susceptible. Of 183 second-generation hybrids, 3 grew the tumor and 180 were nonsusceptible. This result was confusing and unorthodox genetically.

In 1914–16 the writer explained the experimental findings on the theory that the Japanese waltzing mice introduced into the cross from 12 to 14 independent Mendelian genes which were present in *all* F_1 animals, thus producing susceptibility to the Japanese waltzer tumor tissue. In the second generation the random assortment and recombination of these genes would not reproduce simultaneously the total of 12 to 14 required genes, except rarely, for most F_2 animals would be derived from germ cells lacking one or more of the needed genes and these animals would be nonsusceptible. A later experiment using a Japanese waltzer fibrosarcoma gave an F_2 ratio, suggesting that here 4 to 6 genes were involved. This was the Genetic Theory of Transplantation.

Soon after, Strong began working with transplanted tumors of various common (nonwaltzing) mouse strains. He obtained 3-factor, 2-factor, and finally 1-factor ratios. Backcross generations confirmed the genetic theory and it became established and is generally accepted. By the use of this genetic theory various important lines of investigation were opened up. Among them the following may be mentioned as examples.

1) Changes in the genetic nature of tumors themselves were detected and analyzed. These were always in the direction of simplification by the inactivation or by the disappearance of one or more genes. The changes occurred suddenly and irregularly as far as controllable factors were concerned (Bittner, Strong, Cloudman).

2) By selective inbreeding, "single factor" lines were established and the nature of the genes was analyzed (Snell, Gorer). As a result, the genetics of "histocompatability" has been, and is being, determined with great accuracy.

3) The genetic theory has led to the recognition of "enhancing" effects in tumor and in normal tissue growth following transplantation; these enhancing effects are of basic importance to our increasing knowledge of immunological processes.

4) The use of known and controllable genetic stocks and of their tumors has provided the animal material for the established program bioassay of chemotherapeutic agents.

5) The fact that the host animal can sometimes modify the biological nature of the transplanted tumor has been recognized and is being investigated (Barrett).

6) The problem of temporary growth followed by regression of the tumor has been sufficiently defined to make it practical to study and control some of the quantitative aspects of that process.

7) The successful application of the genetic theory of transplantation to normal as well as to neoplastic tissue has provided an experimental method of analyzing strain and individual differences in biochemical composition and in growth phenomena of various organs and tissues as well as the comparison of normal with neoplastic tissue.

8) The rate at which the individual develops its specificity in various tissues and in organ systems can be studied and analyzed by inoculation of alien tissues at various chronological ages.

Transplantation has thus come to be another broad and active field in which biological research will continue to expand and to deepen with fruitful results. It is a major "front" in the war against cancer.

Development of Inbred Strains

In 1909 Johannsen, a Danish botanist, described and explained the production and development of genetically homogeneous "pure lines" of beans by the continued process of inbreeding through self-fertilization. He had previously (1903) seen the possibility of such a technique but did not prove it experimentally until later. In that same year the writer, working under Dr. W. E. Castle at Harvard, had, by recombination of coat-color genes, produced dilute-brown (DBA) and pink-eyed dilute-brown (DBAp) mice. Brother $\times$ sister matings of several lines of each of these varieties was started at once. One of the dilute-brown homogeneous lines survived and is now the DBA/1 strain. It is also the progenitor of the homogeneous DBA/2 substrain.

The process of producing homogeneous genetic strains by eliminating heterozygosity can be successfully carried out by unbroken brother $\times$ sister or parent $\times$ offspring matings. Although the "purification" proceeds more slowly than under self-fertilization, the end result is the same. Strong, by similar methods, produced the albino A strain and the very valuable C3H agouti strain. The writer similarly produced C57BL and turned over C58BL to MacDowell who completed its inbreeding. The C57BR strains A, B, and C were isolated from descendants of a brown segregate in C57BL by J. M. Murray. These strains, together with BALB/c, STOLI, AKR, and others, form the foundation for the great majority of medical research being carried on with inbred mice: Dunning and Curtis, at the Crocker Institute; King, at the Wistar Institute; and Castle, at Harvard, produced and studied inbred rat strains. Eaton and Wright did the same for guinea pigs. Castle and, later, Sawin inbred

rabbits also, though not so closely as was the case with smaller rodents.

At about the same time that the earlier inbred strains of mice were being developed, Slye was publishing a series of papers emphasizing the effect of heredity on the production of various types of neoplasms among the mice in her laboratory. Although the pedigrees of her experimental animals were complicated, she established beyond any doubt: (*a*) that cancer in general occurred much more frequently in successive generations of some families than it did in others; (*b*) that tumors of the *same* type often occurred within certain families much more frequently than within others.

The creation of inbred strains definitely proved that the tendency to produce a given type of tumor could be fixed and maintained in successive generations. This was also evidence that at least some of the etiological factors in tumorigenesis are specific for tumor type and can be separated from one another by genetic selection.

Within a genetically homogeneous strain, transplants of normal or of neoplastic tissue from animal to animal are treated by the host as autotransplants. F_1 animals produced by crossing two inbred strains can grow, either simultaneously or separately, normal or neoplastic tissue from *both* parent strains.

Some of the important lines of research opened up by the formation and utilization of inbred strains are as follows:

1) By the appearance with a high degree of fidelity of the same general types of morphological and physiological development among the individuals and among successive generations of an inbred strain, the investigator knows in advance with a far greater degree of accuracy what to expect than would be possible under any other circumstances or with any other type of material.

2) By exhibiting definite characteristic types and degrees of hormonal action within the inbred strain, experimental manipulation of hormonal balance can give a new method for analyzing the possible role of hormonal action in tumorigenesis or in other biological processes.

3) The high degree of genetic uniformity in an inbred strain provides a "constant" biological unit for quantitation in assaying extraneous agents, or in experimental procedures introduced by the investigator.

4) The reverse technique can be used also with great effectiveness; a single substance or experimental procedure can be tested against a number of different genetic backgrounds of known origin and nature.

Use of these opportunities has already resulted in exciting and stimulating advances in almost every phase of experimental cancer research. There is every reason to believe that they will continue to be an essential element in hastening and extending discovery of new knowledge and will also be the best obtainable tool for repeating, extending, and evaluating experimental procedures in biological and in medical research. The inbred strains and their hybrids used in conjunction with the other methods and techniques described is thus an invaluable "weapon" for future research.

Tissue-Culture Developments

Although interesting and important discoveries in cell physiology and morphology have already resulted from the use of tissue-culture techniques, there remains a great number of exciting possibilities as yet uninvestigated or not sufficiently explored. Among these is the potential value of synthetic culture media in analysis of cell composition and physiology. There should be experimental variation in chemical components not only of adequate synthetic media but also of those media which may reveal selective and significant survival differences, in the response of various genetically controlled cells or tissues. The possibilities in this field of research would seem to be almost unlimited.

More extensive use of exposure *in vitro* of various genetically controlled tissues to different suspected or known carcinogens should, if followed by transplantation into hosts of known but different genetic types and into hosts of different chronological ages, reveal many new facts of importance. These are the sorts of techniques that should help us to *bring into direct observation* changes in tissue response which will in turn aid in analyzing and in eventually understanding the activities of cells and tissues in different types of mammalian individuals *where direct observation is not possible.*

Irradiation Effects and Isotopes

From the more directly biological point of view, the utilization of genetically controlled animals, tissues, and cells offers great opportunities both in quality and quantity of irradiation problems. The work of W. L. and L. B. Russell has already shown the practicality and fruitfulness of this approach. Much of the work with these agents will, however, be in their biophysical and biochemical relationship. The writer is not sufficiently informed in either of these fields to comment intelligently on their possible details of development.

If full advantage is taken of the known techniques of transplantation, inbreeding, and tissue culture, either singly or in combination, the versatility and effectiveness of analyzing and interpreting irradiation effects and the significance of isotope pathways in metabolic and morphogenetic processes will be greatly increased.

Since *beta-* and *gamma*-ray *irradiation* has been well identified as having mutagenic and carcinogenic properties, the relationship between the experimental responses of somatic and germ cells should be further explained by experiments comparing such irradiation effects on the two types of cells.

Up to now the effects of irradiation on the germ cells of mammals have been expressed in mutations which are usually either lethal or which produce morphological abnormalities involving disproportion or other departures from the symmetry and balance of normal development. It is interesting that there is little if any evidence of increased uncontrolled neoplastic growth in the embryos or young produced by the combination or participation of mutated germ cells.

On the other hand, there are in somatic cells many cases of induced neoplasia, which often occur after a long latent period. The biological significance of such prolonged latent periods is not understood and is not receiving nearly so much attention and investigation as it deserves.

Compared with the vast number of cells receiving irradiation in any given area, the number that exhibit a neoplastic change is infinitesimal. If a direct, simple, single process is involved in this change, why do not *all* or at least the great majority of exposed cells show it? The evidence seems to favor a hypothesis of a series or chain of intracellular changes which, in the vast majority of cells, is broken or obstructed before the sequence reaches the tumorigenic threshold. What the trigger mechanisms may be for each step and for each type of tissue are the sort of problems that will require patient and prolonged investigation to match the duration and finality of the processes being studied.

Cell Chemistry and Function

Modern methods of analyzing the chemical composition of cells give real promise of adding knowledge of the differences between cells at various stages of differentiation, of various specific tissues, and even at different stages of functional activity in the same tissue.

It would, however, be well to remember that when a chemical is isolated from the cell and is measured or assayed, the cell itself is no longer dynamic or an integrated living system. This, of course, is not in the nature of a criticism. It is merely a natural technical limitation which is made necessary by the definition of the type of the investigation itself. The same limitation applies to pathology where it will always be impossible to state definitely what the "next stage" of future development would have been had the cell or tissue been left in the living body instead of being fixed, stained, and sectioned.

Studies of cell chemistry and cell function based on isolated material should therefore be integrated with and supplemented by studies on groups of living cells of known and uniform genetic composition *in vivo* or *in vitro*.

Experimental Carcinogenesis

Since the initial discovery of the process of experimental carcinogenesis on the ears of rabbits, there have been literally thousands of experiments with different challenging agents, dosages, sites, strains, species, and ages of animals. Agents have been used alone, in combination, or in sequence. The excellent and comprehensive assembly and tabulation of these experiments by Hartwell will convince anyone of several facts. 1) There is a very large number of chemical agents that can prove to be carcinogenic in one or more types of animal material. 2) There is every reason to believe that the number of such substances will be steadily and significantly increased as time goes on. 3) The action of these substances is now being recorded and assayed under such scattered and uncoordinated conditions that no integrated or progressively analyzable program of procedure, approach, or reduction of variables exists.

There is naturally a question as to when and how far one can extrapolate from animal research to man under any given set of experimental conditions. Some general comments in this connection may be helpful.

The germ cells of higher mammals appear, as a group, to possess most of their basic types of organization in common. The process of maturation, ovulation, fertilization, embryonic developmental sequence, implantation, and parturition are essentially similar in primates, carnivores, ungulates, and rodents, which are the four orders commonly studied.

The internal organization of chromosomes and the behavior of genes in the four orders are closely parallel. It would seem likely, therefore, that a high degree of extrapolation from one order to another is permissible in the field of germ-cell and early developmental studies.

Hormonal origins and behavior are also very much alike in the four orders. Until new evidence of divergence is found, one may compare with considerable scientific justification, experimental results in endocrinology with expectation of fairly close conformity in interorder behavior.

The functions and basic structure of the great organ systems—nervous, digestive, excretory, circulatory, respiratory, skeletal, and reproductive—are essentially the same, although proportional, regional tissue development may vary considerably within an organ or organ system.

The outer epithelial tissues show a considerable variation in structure and response to environmental stimuli. The skin, hair, nails, and teeth are highly specialized; these vary greatly in form and function not only between orders but also between species, strains, individuals, and even between different parts of the same individual.

Experimental response of these structures to various challenges or environmental factors may be expected to be as specific, characteristic, and varied as is the type of animal selected for study.

It is very important to remember this fact, for the speed and convenient experimental response of many of these easily accessible and observable tissues are tempting qualities for the avid and impatient investigator. There is real danger of overextension and unwise generalization from highly restricted and specialized data. This is the main reason why *in order to use such data to their full limit of justifiable extrapolation, the development of a table of standard quantitative responses of genetically controlled animal tissues to known challenges or exposures is an absolute prerequisite.*

With such a foundation available, the value of such tissues for assay may prove to be unique and of lasting importance. *Without* it, observations will remain isolated, fragmentary, and of doubtful usefulness in transfer, extension, or applicability to other work with the same or with different species. It may prove to be impractical to integrate work of this sort; but until the possibility is considered and some concerted and continuing effort at definition and coordination is made, the total of experimental results will remain a hodgepodge of disconnected observations of minimum value as knowledge exchangeable or utilizable between investigators.

It may be well, while waiting for the development of new and more

refined methods, to review the commonly used methods for bioassay of chemical carcinogens and to attempt to determine and to define the variables encountered in the present experimental approach to this problem.

Surface painting is one of the more important and widely used methods of application. *Injection* is another. *Inhalation* (which is often ingestion) is a third. The following discussion applies primarily to the first two methods but also, at least in some of its phases, to experiments using inhalation or aerosol techniques. The first series (I) of six factors, noted below, involves pertinent controllable attributes of the animal or tissue used for assay. The second series (II) of three variables deals with methodology to be considered in broad techniques of application.

I. *Host Response*

A) The *age factor* should be evaluated, both by observation of the total animal and by study of the relative aging of the specifically challenged tissue. This can be done at prenatal, neonatal, and postnatal levels up to and including senescence. Physiological and chronological age do not always develop *pari passu*.

B) The *genetic strain* of animals used should be considered as an aid in the analysis of the carcinogenic effects. Strain response to challenge should be correlated with the known and recorded morphological and physiological characteristics of the strain.

C) The effects of application of the experimental agent should be studied on various *areas of the skin* or on selected target tissues and organs which develop in different ways *structurally* or at different *rates* (according to degree of differentiation, physiological or mitotic activity, repair rate, etc.).

Such studies should lead to additional information on host reaction not only in relation to *histological changes during morphogenesis*, but in relation to certain *genetic influences* which may predictably modify the development of the challenged structures (genes for brachyury, polydactylism, short-ears, hairlessness, etc.).

D) The evaluation of the factors of dispersion and of localization of the agent used should also be considered. This involves application to a defined structure (tail, ear, digit), which can later be removed by surgery or isolated in part or *in toto* by physical (temperature, radiation) or chemical (anesthesia, neutralization) means.

E) Experimental manipulation of hormonal level should be introduced as another means of modifying internal balance and resulting host response. Such studies will contribute not only to our knowledge of the *general* processes of carcinogenesis but also to the possible analysis of recognized sex (gonad, pituitary, adrenal) or developmental differences (thymus, thyroid, and above-mentioned glands), and the detection of new ones.

F) *Manipulation of vitamins and other nutritional elements* as modifiers of the tissues challenged should be a field of investigation. It is

remarkable how little such studies have been used in their broad analytical implications.

II. *Techniques of Application*

A) Possible interreactions of *challenging agents* should be studied by application of individual *agents alone*, in separate *coincidental* or otherwise-timed sequential application, and in *premixed combinations* of various relative proportions of the component agents that are being compared.

B) *Dosage* should be studied and analyzed with the following controllable variables in mind: *total amount* of agent; *concentration* in solvent or other vehicle; *number of applications; time intervals* between applications.

C) All of these variables can be studied *in vivo* and, as the technique of tissue culture and the control of synthetic culture media are developed, they can also be investigated *in vitro*. In this way the broad program of experimental contact-carcinogenesis and of tissue-culture research can be coordinated and used to supplement one another.

It may also be pointed out that any successful efforts to define and agree upon various elements in bioassay of possible carcinogens will have a double value:

1) They will gradually build up a table of standards of reaction for known carcinogens with which assay of unknowns can be quantitatively compared with some prospect of repeatability and continuing significance.

2) They will make possible much greater direct exchange value between investigators of the results which they obtain, thus accumulating at the maximum rate under our present levels of knowledge classifiable and coordinated information concerning carcinogenesis.

3) They will contribute directly to our fund of information concerning the factors within the organism which establish and maintain the internal balances on which health depends and concerning the factors that, by threat to or upset of these balances, predispose to or originate the "constitutional" diseases.

Basis for Future Progress

It would seem that a great deal of progress can be made indefinitely into the future by making deliberate efforts to plan research on the overlapping border problems of the various fields which have been mentioned and to train research personnel who are familiar with two or more of them. For example, virologists who are also trained in genetics, or tissue culture, or irradiation, or isotopes, or cytochemistry, or experimental carcinogenesis would find many chances to discover new facts. So would those skilled in any combinations of these techniques. It would seem that a knowledge of genetics at the strain, individual, tissue, and cell level would probably add to the other disciplines the common basic element of which they were all most in need.

Leaders in organizing borderline research are today few and far between. They have arisen sporadically as a result of their own vision and tenacity in obtaining the necessary training. There should and will continue to be a certain number of them who will appear in the future without special effort or planning and they will continue to be of just as great value as have those who preceded them.

Their number, however, will be pathetically inadequate to take full advantage of the opportunities to expand, deepen, and hasten the increase of our knowledge of the processes underlying the genesis and progress of neoplasia. The existence of these opportunities is realized by only the relatively small group intimately in contact with them. Many of this group are actively and enthusiastically engaged in their own research, jealous of the all too fast passage of time, and painfully aware of the need for expansion of resources to give them the additional "eyes and hands" that would multiply the effectiveness of their efforts.

Few of the active group have the inclination to become missionaries to attempt to convert the powers that hold and distribute sources of increased support. Few can themselves initiate or develop the opportunity for intimate exchange of experiences, views, and plans for the future. A company attracted by the more immediate, practical, and the more glamorous appeal of the clinical or clinically pertinent phases of education and investigation holds the stage and fails to understand that when its *repertoire* reaches a certain point, unless a supply of new basic research knowledge is available, it will have to depend upon "revivals" perhaps with new "orchestration" or "stage settings" to keep the box-office receipts of financial support at a satisfactory level.

Wise patrons of any creative activity, including cancer research, recognize the unavoidable and basic truth that unless the sources of new ideas are developed the evolutionary process of any art or science will cease. The way in which such "wise" patrons are developed is by bringing them into direct or indirect contact with those who are creating. As yet the existence of adequate opportunity for contacts of this sort, in the relationship of the biological sciences to research on both normal and abnormal growth, is conspicuous by its absence or whimsical uncertainty.

The recent demise of the National Research Council's *Committee on Growth* and its replacement by more centralized control of even the recommendatory phases of research support by the American Cancer Society appears to many to be an unfortunate retrograde step. The flowers of polite "recognition" placed on the corpse merely served to emphasize the tragedy of the failure to recognize the potentiality of its value during its life.

Present indications are that the rate of progress of the biological age in cancer research will be "on foot" in the immediate future unless some financial "station wagon" with room to hold the hitchhiking "family" of basic research stops and "picks it up" from the roadside. This seems unlikely for the road on which the "traffic" of project research is buzzing along is broad and level and driving is comfortable. Other hitchhikers

clad in appealing uniforms of "direct service to humanity" are as numerous as service men were at the height of World War II and they do not have the unwelcome "children" of ideas of unproved practical value along with them to require attention to growing and assertive demands for "food" and other resources.

This situation, however, must not discourage those concerned. It is merely evidence of a delay in recognition of true values. It *does not* and *cannot* weaken, mar, or diminish those values. Patience and devotion to ideals and the precious duty of guarding them are an essential part of the duties of those fitted by their nature and training to represent the frontier phases of experimental science.

Having briefly considered some of the major fields of experimental science in which studies on growth are being conducted, we may next suitably discuss certain principles of biology which apply to the problems under investigation. Many of these are tacitly recognized but a summary and review of them may nevertheless be helpful.[4]

Latent Power of Growth

It is the common custom to evaluate the nature of biological processes on evidence obtained by methods that satisfy our senses, with the least disturbance possible.

The growth curve of the mammal shows the most rapid growth *rate* in early embryology with a gradual and reasonably steady decrease in rate until the "adult" stage is reached and progressive growth has "ceased." The differentiated mammalian cell is commonly considered to be a biological unit which has lost at least the greater part of its earlier power of mitosis rate, which its antecedents demonstrated before differentiation. This point of view reached its high point in Cohnheim's theory of "embryonic" rests based on the hypothesis that there were scattered throughout the body a limited and unpredictable number of cells which resisted the restricting process of differentiation and remained in physiological concealment until an opportunity occurred for them to come out of hiding and to go beserk as neoplasms.

Any such conception of the *loss* of power of growth by "normal" mammalian cells overlooks a mass of evidence that indicates perhaps the universal presence of enormous latent power of growth, which is available for use under a number of conditions which call for its expression.

The primitive and most "normal" type of animal cell which has, by an unbroken line of descent, populated the earth with animal life since that phenomenon began, still shows unabated power of growth and reproduction. Studies of protozoa uniformly reveal such power of growth and incidentally but importantly also show that the different *rates* of cell division are inherent functions of various sublines or clones.

From the point of view of biological survival value, therefore, the primitive, rapidly reproducing animal cell is the "normal" unit and the limited

[4] For a more extensive discussion of these principles, the reader is, as before stated, referred to the Lane Lectures given by the writer in 1954 (Stanford University Press).

or restricted differentiated cell is, to a certain degree, an "abnormal" product of aggregation of cells, of division of labor, and of specialized function among them. Because in "higher" animals the vast majority of cells ordinarily obey the control of activity and function superimposed by the tissue, organ, or organism as a whole, we have adopted very literally the evidence of our eyes and speak of "stimulation" when cells resume a power of growth similar to that of their less restricted antecedents. It would be more logical to consider increased growth to be the result of "release" of latent growth energy and to think of processes which are involved in maintaining lower growth rates as "control." Let us see what the body actually does when the internal environment of "control" is challenged by various circumstances.

Regeneration

The striking response of a tremendous increase in rate of cell division by relatively inactive and "controlled" cells following mutilation by incision or amputation is a widespread phenomenon in many invertebrates and in the amphibia. It is hard to imagine a biological "stimulant" as a result of a radical trauma which cannot conceivably create an extrinsic chemical with a stimulatory function. It is also obvious that the cells which regenerate the amputated structure would not have revealed their great innate potentiality of increased cell division had they not been challenged and had the balanced control of their environment remained intact.

Repair

The ability to release latent powers of cell division to repair minor trauma or unbalances is a basic quality in most mammalian tissues. It is the "rule" rather than the "exception" and again bears witness to a great latent power for growth which is so general and nonsensational that we are apt to overlook its great biological significance. Only when the "repair" process fails to cease when the original balance is regained do we recognize the potential energy that is involved. The formation of keloids is a good example of such a situation. Racial, familial, and sex influences, which affect this uncontrolled continuation of "repair" by formation of relatively primitive fibrous tissue, give us further food for thought concerning its deep biological significance.

Replacement

An even less noticeable but perhaps more impressive type of expression of the continuing growth potential of the mammalian cell is the steady process of replacement of worn out or dead cells by the production of new ones. It is a process that while regulated and orderly is continuous throughout the life span and reveals a silent and omnipresent ability to utilize a latent growth ability which is present in essentially all mammalian tissues.

There are other striking examples of latent power of growth possessed by mammalian cells and ready to be used when circumstances require.

Twinning

The ordinary course of development of the fertilized mammalian egg results in the amount of selective and controlled cell division necessary to produce a single complete individual. In some cases, however, a fertilized ovum of the normal size, structure, and chromosomal number will begin to develop into a single embryo which separates into two equal or approximately equal masses of embryonic tissue. Thereafter, *each half* develops into a complete individual which at the close of its period of continuing cell division, culminating at the adult stage, is as large and has experienced just as many cell divisions as would a *single* normal product of fertilization. It is evident that in this case the original fertilized ovum demonstrated its latent ability to develop twice as much tissue by twice as much cell division.

Parthenogenesis

Ordinarily the development of vertebrates depends upon the addition of at least the nuclear material of the sperm to the unfertilized ovum. Experiments by Loeb and Bataillon showed, however, that a certain number of unfertilized frog ova merely pricked by a sterile needle develop into a complete embryo. No chemical was added by this purely mechanical procedure and the logical conclusion is that it released a latent growth potential present but not ordinarily used by the unfertilized ovum.

Neoplasia

This process may reasonably be considered as another form of released latent growth potential beginning *inside* of a cell. It is a change which actually improves the cell as a biological unit, for it divides more frequently than do its neighboring cells and therefore produces more "descendants" in a given period of time.

Internal Balance and Unbalance

The delicacy, accuracy, and persistence with which the animal cell maintains its individual characteristics and reproduces them in its descendants are reflections of an amazing internal balance in both form and function. The failure to reproduce exact replicas is a great rarity under any ordinary environment and any usual challenge. Asymmetrical or unbalanced cells do not, as a rule, produce viable descendants.

This was clearly demonstrated some 30 years ago by Blakeslee and his coworkers who induced chromosomal unbalance in *Datura* by cold and by other experimentally introduced factors. It was observed that plants with $2n$, $3n$, or $4n$ chromosomes were viable and fertile. Those, however, with $2n + 1$, $3n + 1$, or $4n + 1$ were weak and sterile.

It is also the general experience with cancer that while many cells with extraordinary variations in chromosome number and size may be formed in a tumor, those that divide successfully and perpetuate themselves are

usually the cells with a balanced and symmetrical chromosome count which allows successful mitosis, which in turn reflects functional internal balance.

The problem of creating and maintaining internal balance is an essential to orderly progress of life in the cell, tissue, and organ or organism. All of these structures have to utilize cyclic function of some sort. For some, such cyclic function is repeated at relatively uniform or predictable intervals. For others this function may be the response to an unusual or unpredictable challenge of some sort.

There are a number of different levels at which internal balance must be maintained in order to preserve normal function. These levels may be roughly defined as follows:

Intracellular	1) Between components of the gene 2) Between genes in the chromosome 3) Between chromosomes 4) Between essential components of the cytoplasm 5) Between nucleus and cytoplasm
Intercellular	6) Between cells of any tissue 7) Between tissues of any organ 8) Between organs of any organism

At any or all of these levels various influences, either internal or external in origin, may produce unbalance either temporary or permanent.

Examples of the establishment of new centers of balance at different levels are: mutations *within* the *gene*, translocation, other new positional relationships of genes *within* the chromosome, polyploidy or nondisjunctional changes *between* chromosomes, formation of cytasters in the *cytoplasm* of anucleate cells, multinucleate cells or cells with a giant nucleus; nonconforming cells as in mouse mammary glands during the lactation cycle; hyperplasia of the cortical layer of the adrenal in mice and duplication of organs such as polydactylism.

Ordinarily the gene, chromosome, cell, tissue, or organ adjusts itself to the challenge of such unbalance and regains equilibrium around the original center of balance. At times the unbalance is so great or so radical in nature that it impairs and destroys the function of the unbalanced structures or system. At other times the unbalanced structure regains equilibrium of function around a *new* center of balance. There are therefore three general types of possible response to the challenge of unbalance.

The neoplastic process is undoubtedly related to and affected by unbalancing influences of various sorts. The increased rate of cell division is one result, but more important is the independence of certain neoplasms from the control and balance ordinarily maintained between components of a tissue or between tissues within an organ. It has established a new center of balance with a degree of differentiation or lack of it peculiarly its own.

This is a basic reason why much more extensive research on the nature and function of controlling influences in *normal* development and physi-

ology should continue to provide essential new knowledge of what the *release* from such control involves.

A brief discussion of unbalancing influences allowing neoplasia may help to give some idea of the great scope of this process.

Hybridization and Unbalance

This is a problem of parental dissimilarity. The results of combining dissimilar germ cells by fertilization depend upon the degree of dissimilarity between the cells combined. If the dissimilarity is very great *no* reaction occurs and the problem eliminates itself. From this extreme there is, with *decreasing* dissimilarity, a graded series of reactions, some stages of which may be listed as follows:

Decreasing dissimilarity

1) Entrance of sperm—no nuclear fusion:
   - *a*) no development;
   - *b*) parthenogenesis of egg nucleus, no participation by sperm.

2) Entrance of sperm—nuclear fusion:
   - *a*) unsuccessful attempts at mitosis;
   - *b*) impaired or abnormal mitosis;
   - *c*) asymmetrical, impaired, or unsuccessful blastula or gastrula formation;
   - *d*) successful somatic development (sometimes with increased somatic vigor) and growth, no fertility;
   - *e*) same as above with impaired or sex-limited fertility;
   - *f*) same as above but with visible deleterious physiological effects or morphological abnormalities, no influence on fertility;
   - *g*) same as above with no visible deleterious effects on fertility and possible *increase* in somatic growth.

It is in group 2*f* that some extremely significant evidence of the effects of lack of adjustment between different developmental potentialities reveal themselves. Described in order, in terms of the *type of effect*, these same levels may be listed as follows:

1*a*) no effect on release of latent growth potential;
1*b*) destruction of secondary "total cell"—organization control—followed by activity of intracellular growth potential;
2*a*) destruction of "total cell" control and interference by partial and incomplete destruction of intracellular functional "mechanics" control;
2*b*) a lesser destruction and nearer approach to liberation from intracellular mechanics control;
2*c*) destruction of secondary *inter*cellular controls and organizers on the level of early intercellular contacts;
2*d*) impaired ability to control the usual amount of somatic mitosis, and failure to adjust to a point where the internal organization of the condensed potentiality of a balanced and functional germ cell can be accomplished;
2*e*) same as above with production of "unsuccessful" type of germ cell;

2*f*) same as above with *local* failures to establish growth control in balanced and proportional quality or degree;

2*g*) all the proportion-maintaining controls function but at times less rapidly so that more mitoses occur before the final control is established. This delay is due to modified intracellular activity, for the cells retain increased latent growth potentiality or increased power of "insurgency" which later may express itself in *renewed* mitotic activity or neoplasia.

The extensive work of Gordon with hybrid fishes has demonstrated that neoplasia may be a predictable and direct result of unlike developmental patterns attempting to express themselves in the same individual. A similar striking increase in tumor formation over both parent strains was observed by the writer in a species cross in mice.

The metabolic activity of two different genetic backgrounds attempting to adjust to one another may involve competing qualitative or quantitative variations in chemical components or in duration, speed, or sequence of chemical processes. It is therefore entirely logical to expect a greater risk of unbalance at *all* levels when dissimilar developmental patterns are in competition.

A question that will naturally arise is why *inbred* strains with genetic homogeneity may show a very high incidence of neoplasia. Within such a strain the developmental pattern should be as uniform and predictable as one can expect in higher animals.

The answer is that by selection of parents with characteristic genetic tendencies to produce unbalance in certain tissues or organs, these tendencies become genetically *fixed* and recur in the individuals of that particular strain with a high degree of frequency.

If the genetic unbalance occurs in a highly differentiated tissue or organ the localization of the neoplastic process is more complete, as for example in mammary or pulmonary adenocarcinomas. If the tissue is widespread and relatively undifferentiated, as for example connective tissue, the location of the neoplastic change is less predictable, as for example in fibrosarcoma formation.

Hormones and Unbalance

The first example of clear-cut experimental evidence of hormonal influence on tumor formation was published by W. S. Murray (1927). The DBA strain of mice with which he was working was regularly producing about 80 percent mammary tumors in breeding females and from 35 to 40 percent in virgins. No mammary tumors were produced by males.

Recognizing the possibilities of successful homologous transplantation within this inbred strain, Murray placed the ovaries of sister animals in castrated males. The tumor incidence in these "feminized" males was essentially the same as that in virgin females. This demonstrated the influence of the intact ovary on mammary-tumor formation.

Soon after Murray's work, Lacassagne, utilizing the follicular hormone and diethylstilbesterol, obtained results similar to those resulting from transplantation of the intact ovary.

Later work by Strong and others at the Jackson Laboratory demonstrated that different inbred strains had quantitatively distinct degrees

of follicular hormonal activity. Thus while strain C3H produced the same tumor incidence (90± %) in breeding females and in virgins, strain DBA had approximately 80 percent ± and 30± percent, respectively. Strain A, however, produced 80 percent ± and 5 percent. Hummel in studying the suitability of the A strain for Aschheim-Zondek pregnancy tests found that its level of follicular hormone secretion was so low that there was an uncertain and unreliable response.

Beginning in 1939 an even clearer and more specific series of results were obtained, by Woolley, Dickie, Fekete, and the author, on interhormonal action in relation to mammary and to adrenocortical neoplasia.

When the gonads were removed from neonatal mice of three distinct inbred strains (C57BL, DBA, and CE) each strain gave a different characteristic and consistent response.

The C57 mice behaved like the classical examples of castration effect. Relatively underdeveloped genitalia and secondary sex characters and no discernible interhormonal reaction by compensatory activity was the rule.

In the DBA mice, after a period of inactivity, there was evidence of resumed development of female secondary sex characters including mammary-tissue growth and the appearance of mammary neoplasms. Examination of the adrenals showed hypertrophy and hyperplasia of the adrenal cortex. Irregular blunt "fingers" of hypertrophic and hyperplastic cortical cells grew down toward the medulla. These outgrowths histologically bore some resemblance to ovarian tissue and were undoubtedly the source of the "feminizing" secretions.

In the CE mice, again after an inactive period, distinct "masculinization" effects were evident. These included renewed male-type growth of genitalia, attempted copulatory behavior, etc. Within a few months adrenal nodules were grossly palpable. Later histological examination showed in *every case*, adrenocortical carcinoma with little or no discernible glandular structure. These carcinomas were readily transplantable into CE mice and when placed in castrated animals of either sex produced marked signs of masculinization.

More recently, by early gonadectomy, Dickie has released, in certain genetic types of mice, activity of the pituitary resulting in hyperplasia and neoplasia. This response is predictable and controllable.

There is, therefore, overwhelming evidence that gonadal-adrenal-pituitary balance, which normally determines and regulates the hormonal growth-controlling activity of these three glands, can be experimentally upset by "deprivation" technique; the results of the "upsets" are constant, predictable, and striking.

It is entirely logical to assume that if a major and critical unbalance can be produced by the "all-out" experimental deprivation procedures, there are minor strains and challenges toward unbalance produced by impairment or diminution of hormonal production or function under the conditions which occur "normally" in the body. There are many examples of experimental evidence which bear directly on this conclusion.

A number of simple Mendelian genic mutants in mice and other mammals show established differences in hormonal activity which markedly affect general or local growth. Dwarfism and anemia are examples.

Less simple but still clearly determined genetic differences appear in cyclic phenomena such as the reaction of mammary tissue to the estrus and pregnancy sequences. Here Fekete has demonstrated ovulation and corpora lutea differences between intact C57BL and DBA mice. The histology of the mammary tissue during pregnancy is also characteristic of each strain.

Many growth changes including hyperplasia and neoplasia have also been recorded by various investigators in response to unbalances experimentally induced by excess or deprivation of hormones.

It would appear that the influence of hormonal unbalance upon the incidence of certain neoplasms is well established and that the future is likely to reveal that this relationship is widespread, varied, and highly significant.

Viroids and Unbalance

Reference has already been made to the work of Rous and others who demonstrated the importance of a virus in the etiology of avian sarcomas. Shope, Bittner, and Gross are among those who have observed the same general type of etiological factor in mammals.

The important fact to remember is that the entrance into the cell of a growth-influencing viroid, produces an unbalance between nucleoid and cytoplasmic cellular components. It is also evident that this process is not pathogenic when neoplasia (increased mitosis) results. The "modified" cells often retain, completely, the biochemical specificity of the organism in which they occur. At times the specificity is lost to a greater or lesser degree but such a loss is not a *sine qua non* of neoplasia due to viroid-induced unbalance. The mitosis-influencing viroid is, therefore, "at home" in the cell and arouses no harmful disturbance of organization or function. In this respect it does not differ from plasmagenic type of activity.

There have been a number of interesting and stimulating discussions of the parallelism and perhaps similarity between genes and viruses, and the field of viroid participation in neoplasia will be a fruitful contributor to the further elucidation of such relationship and of other basic biological processes.

Unbalance of Intracellular Components

The facts that cyclic unbalance within the cell is the chief characteristic of the process of cell division and that the rate of mitosis and its control, or lack of it, are basic parts of the neoplastic process indicate that somewhere within the boundaries of this field—widely defined—should be found the answers to or the major leads toward the explanation of the origin of cancers and other tumors. There are various levels of organization of components within the cell at which unbalance may occur. They can be roughly considered as follows:

Nuclear-cytoplasmic unbalance.—Reactions between nucleoid material and cytoplasm are, of course, one of the essential and basic types of intracellular activity.

The origin and function of plasmagenes, the degree of permanence of changes induced in the cytoplasm, macronucleus, and micronucleus and the spread of gene products through the cytoplasm and through cell membranes are evidence of the complexity and orderliness of intracellular function.

Mechanical modification of the proportion of nuclear to cytoplasmic material has produced interesting and important changes in cell function reflected in the mitotic process. Heat, cold, colchicine, X rays, radium, and chemical carcinogens are among the agents that have been used experimentally to modify existing "normal" nucleo-cytoplasmic balance within the cell. Various general and/or characteristic responses affecting type and rate of mitosis have been observed.

Intracytoplasmic unbalance.—Although comparatively few investigations have been carried out in this field there is evidence that it is an area of research worthy of far greater emphasis. Changes in the proportion of mitochondria in the cytoplasm are believed by some to be associated with anaplasia and the degree of independence and growth in neoplasia.

Anucleate cells in *Artemia* and in *Triton* can divide successfully for at least a time. This indicates that the cytoplasm has, in its own right, latent potentialities for activity in cell division as yet little understood, and of great scientific interest.

The development of a combination of microdissection and tissue-culture techniques should give new and interesting knowledge of intracellular processes affecting cell division and therefore bearing on neoplasia.

Intranuclear unbalance.—The most important and most studied intranuclear structures are, of course, the chromosomes. Their constancy in number, form, and genic structure makes it possible to recognize and record divergencies from the normal structural conditions and from normal function as well.

Reference has been made to the existence of changes in chromosome number, which may be balanced (tetraploidy, triploidy, haploidy) or unbalanced. Boveri's theory of cancer origin considered the greatly increased numbers of chromosomes seen in certain cells of some neoplasms as the etiological basis for uncontrolled mitosis. The later observations that the fastest dividing and most "successful" neoplastic cells often have the usual $2n$ chromosome count and that many cells with large numbers of extra chromosomes are not neoplastic would seem to invalidate the latter phenomenon as the *cause* of cancer. It is more likely that the exuberance of mitosis, released when neoplastic growth occurs, may produce unusual or new cell types of many sorts including hypernormal chromosome content.

Differences in relationship *between* chromosomes, such as nondisjunction, fractionation, translocation, etc., are well known. They may be induced by experimental processes, such as cold, irradiation, or chemicals. They

are also subject, in some cases, to the influence of one or more genes. They are often followed by abnormalities in growth and/or structure. *Within* the chromosome the balanced relationships between genes can also be upset by various experimental procedures. This is a third level of intranuclear organization where the principle of balance can be investigated.

Finally within the gene itself there exists a high degree of constancy in substance and organization. In the vast majority of its reproductive efforts the gene forms an exact replica of itself with amazing effectiveness. Occasionally, however, a rearrangement of its molecular structure appears as a "mutation" and the "new" type of molecular organization then reproduces itself with the same order or accuracy as did the old type that preceded it. The fact that many of the known carcinogens have also the power to increase mutation rate has led to the considerable popularity of the mutation theory of cancer causation.

While it is probable that in some cases intrachromosomal change (mutation) may be involved in changing the mitotic rate of the cell, it seems equally probable that other intranuclear, cytoplasmic, or combined unbalances can result from variation in amount or in activity of other cellular components than the genes.

There is nothing mutually exclusive about the concept of coexistence of many causal possibilities and circumstances in the origin of neoplasia. Cancer may arise in almost any body tissue and the tendency for it to appear in certain sites may be increased and maintained by genetic selection. Tissues are balanced and controlled in many ways. Their cells are often in very different environmental relationships to one another. Release of control may well have many different origins and effects. It would indeed be surprising if this were not the case.

Release of Control

In the establishment and maintenance of functional balance in any living unit it is obvious that the establishment of *intra*cellular organization must have been the first order of evolutionary progress. Only in this way could the single-celled organisms have preceded and made possible multicelled forms.

Secondary balance *between* cells must first have been of a type that maintained *contact* between individual cells. At the same time a high degree of independence and of complete functional capacity of the individual cell must have been necessary because of the "looseness" of affiliation. Later, more complicated controls of functional balance between tissues and organs which resulted in more complex and complete division of labor between cells and tissues and organs must have appeared in the evolutionary chain.

When the organism is challenged by unbalancing agents for which it has no established defense, it is logical to expect that the more recently acquired and accessory types of "controls" would be the first to be impaired or de-

stroyed. The result of such impairment or destruction on intertissue or interorgan "controls" would be the release of the latent powers of growth which these "controls" had held in check.

It may well be that the "increased" growth following "whole-body" irradiation with small doses is the result of the *destruction* of such more complex and vulnerable "secondary" intercellular "controls." This may give the misleading impression of "stimulation" of growth when actually only the release of the latent *intra*cellular activity is involved by the destruction of intercellular restrictions.

With increased exposure (dosage) the destructive process would affect the *intracellular* balance first by upsetting the interrelations of cellular components, and then by destroying the basic physical-chemical molecular and atomic structure. Thus, the series of effects of irradiation would first give the appearance of "stimulation" of growth by destruction of *secondary* balance controls, thereby releasing the latent potential for growth. The continuation or intensification of its destructive activity would then break down *primary* controls and impair or destroy cell function and finally cell organization and structure.

It would seem that a hypothesis or explanation of this type was more logical, simple, and consistent than one that, believing all we *see*, accepted as direct *stimulation* the increased growth activity of small doses of an unbalancing agent, which in larger doses was admittedly destructive and lethal. The question can fairly be asked, "what does the small dosage of a physical agent, affecting molecular and atomic structure and arrangement, add as a stimulant to processes already perfected by evolutionary selection?"

The proposed hypothesis also brings into a consistent and similar relationship the effects of other chemical (carcinogens), biological (hybridization), endocrine, viroid, and other growth-affecting influences. In many cases, light exposure to such agents may release growth potential—if any effect is noted—and in massive doses they can impair or destroy life organization and function.

Conclusion

It is evident that if the origin of cancer involves delicate intracellular changes we must achieve certain advances before we can hope to understand either the changes themselves or the essential steps which lead up to them.

The nature of the cell must be known and controlled in terms of genetic origin, nutritive requirements, and potentiality for division. In order to analyze the process we must be able to observe it and its descendants both *in vitro* and *in situ* (by indirect appraisal of its functions), and by transplantation *in vivo* into sites deliberately chosen and into individuals of known genetic composition.

The nutrient media of the cell must be capable of synthetic control *in vitro* and of measurable experimental modifiability *in vivo*. Any lesser degree of control will involve the existence of variables which may

prevent complete analysis and which will certainly limit and obscure the extent to which results can be applied in a general way.

There are certain fields of biological research which must be far more completely organized and extensively developed before we can complete the necessary foundation for continued and progressive increase in essential knowledge. Among these may be mentioned the following:

1) Maintenance of a large number of genetically known strains of normal and cancer cells and of a wide variety of neoplasms by transplantation both *in vivo* and *in vitro*.

2) Development of standard tables of quantitative potency relationships of known carcinogens for use in assay of tissue changes and of suspected agents.

3) New and improved techniques of manipulating both unfertilized and fertilized mammalian ova so that their development *after* experimental challenge may be accurately and conveniently compared with the normal developmental processes.

It is admitted that such a conclusion is not encouraging to those, and there are many, who allow ready optimism, impatience, or material expediency to lead them to underestimate the complexity of the problem. On the other hand, the honest recognition of the extent of one's ignorance and of the scattered immaturity of our present attack, in spite of its increased volume and intensity, is the first step on the road to the acquisition of knowledge essential to the final solution.

Continued exposition and emphasis of this complex situation is an unavoidable duty and perhaps the best contribution remaining to those of us who have seen the early contacts with the "enemy" develop into the full-scale conflict from which final victory will be won by those who follow.

Cytological Studies of Tumors. XXI. A Comparative Ideogram Study of the Yoshida Sarcoma and Its Subline Derivatives [1, 2, 3]

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During the last few years, ascites tumors of rats and mice have provided favorite material for various types of cytological analyses of tumor cells, which has resulted in an accumulation of valuable data toward understanding the mechanism of tumor development. The Yoshida rat sarcoma was the first subjected to chromosomal analysis. In 1951, Makino (*1*) published a paper on the chromosomes of this tumor, and since then other investigators have done considerable work with valuable findings (*2–9*). The technical difficulties in earlier studies, however, considerably hampered the chromosomal analyses of both ordinary and neoplastic cells of rats and produced wide descrepancies in results. Progress in the solution of these difficulties in later years has begun to resolve these contradictions. Yosida (*4*) dealt with the origin of the V-shaped chromosomes in rat ascites tumors using his improved squash technique. Very recently, Tjio and Levan (*2*) made a comparative ideogram analysis of the rat and the Yoshida sarcoma by applying their orcein-squash method, inquiring in detail into the morphological differences in the chromosomes of the tumors and those of the host, bearing in mind that the development of tumors might be reflected in chromosomal changes.

In the course of the cytogenetical studies of tumors carried out in the authors' laboratory, 4 chromosomal sublines that were derived from the Yoshida sarcoma have recently been found. These sublines are clearly distinguishable from one another, and also from the original stock tumor, by several cytological characters. The greater part of this study was devoted to a chromosomal comparison in these 4 sublines of the Yoshida sarcoma with the use of an improved water-pretreatment squash technique. The data obtained emphasize that chromosomal changes are important in the development of a new tumor type or that changes in tumor type take place in close association with genotypic changes.

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Material and Methods

Tumor samples derived from the Yoshida sarcoma and its 4 subline derivatives, tentatively designated as sublines A, B, C, and D, furnished the material for the present study. The material of the Yoshida stock tumor for examination was obtained from early transfer lines, during the period 1950–52, and, in 1955, from a transfer line maintained by Dr. R. Kinosita, City of Hope Medical Center, Duarte, California. Subline A was produced by Hamazaki *et al.* (*10*) by means of intraperitoneal injections of the filtrate of the Yoshida sarcoma (probably stock tumor) that was prepared with the use of a Seitz-EK apparatus. There are no precise accounts on the origin of subline B, except that it was originally derived from stock tumor during serial transfers in the laboratory of Dr. Tomizo Yoshida, University of Tokyo, but the date of derivation is not clear. The survival of this subline seems to predominate over that of the stock tumor. Rough cytological examination shows that subline B apparently was distributed to many medical laboratories in Japan. In May, 1955, a line was transferred from Dr. Yoshida's laboratory to that of Dr. Katsuo Takeda, Medical School, Hokkaido University. The material for the present study was sampled from tumor animals at the 30th, 33rd, and 35th transfer generations of this transfer line which has been maintained in Dr. Takeda's laboratory. Subline C was developed by Hamazaki *et al.* (*11*) through feeding the Yoshida sarcoma (probably stock tumor) to nontumorous rats. More striking is the origin of subline D: This line was derived from subline C by intraperitoneal inoculation of refrigerated tumor cells in the authors' laboratory. After cold storage at −2° or −1° C. for 6 days, the ascites tumor was injected into new rats; 1 out of 12 developed a tumor subline whose cells are characterized by a new ideogram.

For observation of the chromosomes, a water pretreatment method was employed advantageously. To droplets of ascites' suspension placed on slides was added a nearly equal volume of tap or distilled water, and after 5 to 10 minutes the suspension was stained with acetic dahlia. Squashing was done by applying an even pressure on the coverslip with a finger. As a result of the pretreatment and pressure, the chromosomes were more or less straightened; furthermore, both the centromeric region and the splitting between the chromatids were considerably exaggerated in the individual chromosome. The somatic chromosomes of rats were investigated by means of the tissue-culture method in combination with a hypotonic solution (*12*). The chromosomes of the male germ line also were investigated with the water-pretreatment squash method. Pieces of testis were transferred to distilled water for 15 minutes and then subjected to the acetic dahlia squash technique as described.

Results of Observations

Chromosomes of Ordinary Tissue Cells of the Rat

Before investigating tumor chromosomes it is important to have exact knowledge of the chromosomes of ordinary tissue cells of rats. The appli-

cation of modern techniques by recent investigators permits an increasingly accurate analysis of the rat chromosomes (*2*, *4*, *13–15*). Most recently, Tjio and Levan (*2*) gave a full ideogram analysis of the rat with their improved orcein-squash method. The ideogram given by Tjio and Levan shows a close approximation, though not identical, to that observed in the recent samplings of our material.[5] The results of the present analysis are summarized as follows (figs. 1–3): In the 21 pairs of the somatic complement there are 7 autosomal pairs provided with a median-submedian centromere, 5 autosomal pairs with a subterminal centromere, and 8 autosomal pairs with a terminal centromere. The X seems to be subterminal and approximates the third autosomes in length. Tjio and Levan (*2*) have reported that the X is rod-shaped and is probably between the fourth and fifth autosomal pairs in size. The Y is represented by the smallest one of rod type. It is still questionable whether all of the telomitic elements are actually terminally attached because occasionally some of them show constrictions close to their ends. As discussed by Ohno and Kinosita (*15*), there is a possibility that further technical improvements may produce evidence that almost all elements of the telomitic set are of acrocentric nature.

Chromosome Numbers of the Yoshida Sarcoma and Its Subline Derivatives

Earlier studies have revealed that the chromosome number of the stem cells in the Yoshida sarcoma varied around 40, with rather gradual fluctuation both above and below that number (*1*, *5*, *6*, *16*). In the early phase the cells with the modal number were 40 to 50 percent in frequency, whereas the most recent samplings showed an increase in frequency of the modal cell to as much as approximately 70 percent. This may be an indication that the nucleoplasmic balance in relation to the activity of the tumor cell has increased with successive transfer generations.

Four subline derivatives of the Yoshida stock tumor considered here are all of diploid type (table 1). The frequencies of the cell of diploid range were found to be 96.6, 93.2, 94.4, and 96.9 percent for sublines A, B, C, and D, respectively. Exact chromosome number was obtained on

TABLE 1.—*Frequency distributions of diploid and hyperploid cells in 4 sublines and a single-cell clone of the Yoshida sarcoma*

Tumor	Diploid cells ($\pm 2n$)	Hyperploid cells ($> \pm 3n$)	Total
Subline A	227 (96.6%)	8 (3.4%)	235 (100%)
Subline B	205 (93.2%)	15 (6.8%)	220 (100%)
Subline C	153 (94.4%)	9 (5.6%)	162 (100%)
Subline D	247 (96.9%)	8 (3.1%)	255 (100%)
Single-cell clone from subline A	322 (94.3%)	20 (5.7%)	352 (100%)

[5] The ideogram reported by Makino and Hsu (*12*) and that of Sasaki presented in Makino's review (*3*) are to be revised in this report.

TABLE 2.—*Chromosome number distribution around the 2n mode in 4 sublines of the Yoshida sarcoma*

Tumor	Transplant generation	Chromosome number										Total
		35	36	37	38	39	40	41	42	43	44	
Subline A	78–81	—	1 (0.8%)	4 (3.2%)	7 (5.6%)	84 (67.2%)	27 (21.7%)	1 (0.8%)	—	—	1 (0.8%)	125 (100%)
Subline B	30–35	—	—	—	3 (3.0%)	6 (5.9%)	62 (61.4%)	11 (10.9%)	12 (11.9%)	7 (6.9%)	—	101 (100%)
Subline C	10–26	—	5 (2.8%)	5 (2.8%)	76 (42.2%)	61 (33.9%)	29 (16.1%)	3 (1.7%)	1 (0.5%)	—	—	180 (100%)
Subline D	2–5	—	—	1 (1.1%)	9 (10.0%)	20 (22.2%)	46 (51.1%)	9 (10.0%)	5 (5.6%)	—	—	90 (100%)
	13–27	1 (0.7%)	5 (3.6%)	8 (5.8%)	21 (15.1%)	78 (56.1%)	23 (16.5%)	2 (1.5%)	1 (0.7%)	—	—	139 (100%)

the basis of 50 to 70 metaphase plates in which accurate count was possible, with the material obtained at the 2nd and 5th day after transfer. Table 2 shows the frequency distribution of chromosome number around the diploid mode. The frequency peaks of the 4 sublines under consideration exhibit a clear difference from one another. The modal value falls in 39 (67%) for subline A, 40 (61%) for subline B, and 38 to 39 (75%) for subline C, respectively. In subline D, however, the modal number was 40 (51%) in the second to fifth transfer generations, while it was shifted toward 39 (56%) in later generations.

The evidence shows that each subline has a characteristic stem-cell number mode and distribution of chromosomes, though there is a rather wide range in the variation of chromosome number.

Chromosome Morphology of the Yoshida Sarcoma and Its Subline Derivatives

Yoshida stock tumor.—Makino (*1, 5, 6*) and Makino and Kanô (*16*) have reported that the stemline ideogram of the Yoshida stock tumor differs greatly from the normal ideogram of the ordinary tissue cells by containing certain numbers of V-, J- and rod-shaped elements, among which a prominent V-shaped element of outstandingly large size is very remarkable. Figures 4 and 5 are two representatives of the ideogram of the Yoshida stock tumor from the most recent samplings (1955). In each, the occurrence of a V-shaped element of prominently large size is remarkable and grossly distinguished the Yoshida stock tumor from the normal ideogram of the rat.

On comparison of the ideograms between the rat and the Yoshida stock tumor, a general likeness of chromosomes is noted, but closer observations make it clear that the likeness is merely superficial and that most of the chromosomes of tumor cells differ either in shape or size from those of normal cells. In the present study, therefore, it was rather difficult to arrange the tumor chromosomes into homologous pairs in conformity with the view expressed by Tjio and Levan (*2*). It is apparent from this that numerous structural changes have occurred in the tumor chromosomes in the course of the development of the tumor since its origin. No positive morphological evidence has been obtained for the origin of the big V chromosome. The same may be said for a few small two-armed chromosomes. It is, however, supposed that certain rearrangements may cause the change in locus of centromeres in chromosomes.

The results of these observations tend to substantiate the conclusion of Tjio and Levan (*2*) that a great many changes have taken place during the development of the tumor and not just a few gross changes that are especially obvious. At the same time, there must be a revision of the earlier opinion expressed by Makino (*1, 5, 6*), based on observations by the older technique on the chromosomal relationship between the tumor and normal tissue.

Subline A.—The stemline ideogram of this tumor is provided with two distinct V-shaped elements of remarkably large size, which grossly

distinguish this tumor from the ordinary tissue, and also from the stock tumor.

Out of excellent metaphase plates, which permit exact examination of chromosomes, five examples are presented for reference (figs. 6–10). The chromosomes were arranged according to an approximate order of size. Two outstandingly large V's are placed out of rank. The members in each of the serial alignments do not, therefore, indicate homologous mates. The lengths of the 2 large V-shaped chromosomes were measured on the basis of 15 metaphase plates drawn at the same magnifications, and the average value was obtained for comparison (table 3). It is evident after comparison that the 2 large V elements slightly differ in size; the average length is 6.4μ for a larger V and 5.5μ for a smaller V. At present it is safe to state that the origin of the 2 V chromosomes remains unknown because there is no positive evidence available for its explanation. The cytological distinction of this tumor is not limited in the 2 large V's.

TABLE 3.—*Average lengths of large V-shaped chromosomes in 4 sublines of the Yoshida sarcoma*

Tumor	V	Avg. length (μ)	No. of cells observed
Subline A	V_1 V_2	6. 4 5. 5	15
Subline B	V_1 V_2	7. 0 5. 7	15
Subline C	V_1 V_2 V_3	6. 8 5. 8 5. 6	15
Subline D	V_1 V_2 V_3 V_4	6. 7 5. 8 5. 2 5. 0	15

Close analysis provided evidence indicating that most of the tumor chromosomes are not completely identical with the somatic chromosomes, either in shape or in size, though there is a superficial likeness between them. Furthermore, it seems very difficult to identify homologous pairs in the tumor chromosomes because each seems to have undergone a great change, or changes, during the development of the tumor. The situation is thus similar to that of the stock tumor.

With the technical aid of Mrs. Y. Hisada, the single-cell inoculation was undertaken in subline A. Positive results were obtained in 7 of 20 trials. Cytological observations have revealed that the single-cell clone was similar to the original subline A in several cytological characters. The chromosome count showed a mode of 39 with a frequency at 60 percent. The chromosome patterns were also similar between the original tumor and its clone. The results of this incidental study strongly support the existence of the stem cell.

The cytological evidence, as noted, clearly indicates that subline A is a cellular descendant from the stock tumor. This subline was maintained for over 140 generations in serial transfer from October 1954 to June 1956.

Subline B.—The neoplastic population of this tumor is nearly identical with that of subline A in that the stem line consists of cells which are grossly marked by 2 large V-shaped chromosomes of similar feature (figs. 11–15). It is seen from table 3 that the 2 large V-shaped elements are unequal in size; the average length measured on the basis of 15 good metaphases is 7.0μ for the larger V-shaped chromosome and 5.7μ for the smaller V. General comparison of the ideogram between sublines A and B indicates a general similarity, but closer examination reveals some gross differences. Especially obvious is the difference in the size of the large V's. The larger V of subline B slightly exceeds in size the corresponding one of subline A, though the smaller V's of the 2 sublines are nearly equal in size (table 3). In this case, also, the authors have no decisive morphological evidence for the explanation of the origin of the 2 big V chromosomes.

Subline B has been serially transferred for over 100 transfer generations, from December 1955 to March 1957, with its own stemline chromosome pattern.

Subline C.—This subline is characterized especially by the tumor cells containing 3 characteristic V-shaped elements of prominently large size (figs. 16–20). These elements clearly distinguish the tumor cells of this subline from those of the stock tumor and also those of sublines A and B. After comparative study of the lengths of the 3 large V's it was found that the largest V element differs in size from the other 2 V's, which are apparently identical in length. The average length obtained from 15 metaphase plates was 6.8μ for the largest V, 5.8μ and 5.6μ for the other 2 V's (table 3). From the above data it may be speculated without positive proof that one of the smaller V's may arise through nondisjunction of another V, as the latter 2 are nearly equal in size and shape. Although the chromosomes except for larger V elements show a general similarity between sublines A, B, and C, closer comparison makes it apparent that they are not completely identical in shape and size in the 3 sublines. It is most probable that there may be in the chromosomes some structural or mutational changes which may have led to this new type of tumor.

The cytological feature obtained for subline C clearly shows that this tumor is a cellular progeny of the stock tumor. This subline has been maintained in serial transfer for over 130 generations, from August 1955 to March 1957, without visible shift in its stemline ideogram.

Subline D.—Of great interest is the rise of subline D. This line developed in a rat that received an intraperitoneal injection of tumor cells of subline C which had been subjected to freezing. This tumor is characterized by a population of neoplastic cells with 4 V-shaped chromosomes of remarkable size. By this gross difference this subline is clearly discernible from the stock line as well as from the other sublines including C, which is the predecessor of this tumor (figs. 21–25). It cannot be definitely con-

cluded that the cold storage of tumor cells directly caused the chromosomal change leading to the rise of the new subline D. The average lengths of the 4 large V-shaped elements were obtained as 6.7μ for the first V, 5.8μ for the second, 5.2μ for the third, and 5.0μ for the fourth (table 3). Comparison of the data shows that 1 of the 4 V's is apparently larger in size than the other 3. Out of the latter 3, one (the second V) is larger in size than the other 2—the third and fourth V's which closely approximate each other in size. As with subline C one may speculate that the fourth V may originate from the third V through its nondisjunction. This view is based merely on the relative magnitude of the V chromosomes and on the modal number of chromosomes characteristic of this subline.

Subline D and C are generally alike in respect to the chromosomes other than the 4 larger V's, but closer comparison reveals dissimilarity between them in shape and size of most chromosomes. This indicates that shifts may have taken place in more or less degree in the individual chromosomes of subline D during the course of its development from subline C.[6] It is interesting to see the deviation and rise of one subline from another that has taken place in association with the change of chromosomes, probably involving structural rearrangements and gene mutation.

Tissue pieces from the solid tumors, which were produced through subcutaneous transplantation of subline D of ascites form, were cultured according to the roller-tube method, and the chromosomes were observed in dividing cells in the culture material and compared with those observed in the original ascites tumor before subcutaneous transplantation. Comparison revealed a close similarity in the chromosome pattern, both in morphology and in number, between the samples taken from the culture material and those from the ascites tumor. Figures 26 and 27 (two ideograms obtained from cells in the culture material) will supplement these statements. The maintenance of the 4 V stem line is thus evident.

Subline D has been continued for over 90 generations (March, 1957) in serial transfer since its first appearance in January, 1956.

Discussion

Recent investigations on the chromosomes of transplantable ascites tumors of the rat and mouse have presented important data that support and strengthen the hypothesis of the stemline cell from which the tumor propagated (*1*, *2*, *5–8*, *16–32*). There no longer seems to be any doubt that the tumor cells most frequently occurring with characteristic chromosome-number mode, together with particular ideogram, form a stem lineage (or lineages) of tumor cells which contribute principally to the neoplastic growth. Each neoplastic population is characterized by a stemline ideogram (or ideograms) by which the tumor is clearly distinguishable from other tumors, as well as from the ordinary tissue. Apparently the genetic constitution of a tumor is closely correlated with

[6] It is interesting that subline C and D are dissimilar in respect to immunological relation and transplantability. For example, subline C is wholly nontransplantable to rats of Fischer strain, while subline D shows a high transplantability—83 percent to rats of the same strain. The details will be reported elsewhere, by the junior author, together with other related evidence.

the chromosome constitution of its stem cells, and therefore it is the stem cells that maintain and determine the genetic pattern of each tumor. However, this argument seems to give the impression that the specific ideogram different from the normal ideogram is a necessary condition for malignancy. It should be mentioned that some tumors, such as 6C3HED, MTK-sarcoma III, and certain carcinomas, show no remarkable difference in chromosome from normal tissues (*28*, *31*, *33*, *34*). Even in such cases, however, the likeness may be merely superficial, because there may be many structural and mutational changes in the tumor chromosomes which may be associated with the neoplastic condition. Probably, the specific ideogram of the tumor is correlated with malignancy and with the adaptability of the tumor cells to new circumstances.

Very recently, Tjio and Levan (*2*) made a comparative ideogram analysis of the rat and the Yoshida sarcoma in order to define the morphological differences between the chromosomes of the tumor and those of its host, as the development of tumors is reflected in chromosomal changes. Thanks to this close investigation, the chromosomes of the ordinary rat tissue have been fully analyzed in comparison with the chromosomes of the Yoshida sarcoma. Their conclusion is that the evolution of the tumor ideogram cannot have taken place only as a result of new combinations of unchanged chromosome arms, and that a great number of structural rearrangements must be assumed. Our general conclusions, the results of an improved technique, tend to substantiate the idea and evidence presented by Tjio and Levan (*2*); at the same time, the early view of Makino (*1*, *5*, *6*) based on observations by the older methods on the chromosomal relationship between the Yoshida sarcoma and the rat is revised here (figs. 28–35). It has been shown after comparison of the chromosomes of the Yoshida sarcoma and those of the ordinary tissue that most of the chromosomes of neoplastic cells differ in shape and/or size from those of normal cells. With the new material prepared by improved technique, the authors find difficulty in identifying the homologous mates in the tumor chromosomes and in arrangement of them into pairs. It seems likely that the change in locus of the centromere in a chromosome may be caused by rearrangement in the chromosome. It is then assumed from the above evidence that there may have been in tumor chromosomes considerable structural and mutational changes involving rearrangements and small adjustments that may correlate with the neoplastic condition. Apparently the changes are not only the few gross ones determined by the V-shaped chromosomes of prominently large size.

The main interest of the present study concerns the analysis of the stemline ideogram in 4 subline derivatives from the Yoshida sarcoma, with particular reference to the distinction and rise of sublines in tumors. As given in the foregoing descriptions, this investigation has established, after chromosomal analysis, the distinction of 4 sublines in the Yoshida sarcoma. Each subline is generally characterized by its specific stemline ideogram, which is grossly distinguishable from the others by the occur-

rence of 2, 3 and 4 V-shaped chromosomes of markedly large size, as well as from the stock tumor which is characterized by one such V chromosome. There are 2 V-shaped elements that characterize each subline ideogram for sublines A and B, 3 for subline C, and 4 for subline D. It seems probable that the V-shaped chromosomes here under consideration seem not to be identical in nature and origin in the 4 sublines. At present it seems better to state that the origin of the large V chromosomes remain unknown, because there is no positive morphological proof for its explanation, while a speculation involving the nondisjunction of the V element was applied with a slight morphological support to one of the V's occurring in sublines C and D. The view on the origin of the large V's due to a centric fusion between two rod-shaped elements through translocation, though it was expressed in the former papers by Makino (*3*, *9*), is merely hypothetical.

In addition to the gross changes, as represented by the large V chromosomes, there are many other differences between each of the 4 sublines, both in the structural features of chromosomes other than the large V's and in the modal chromosome number. Furthermore, it is noticeable that the subline derivatives are somewhat different from one another as well as from the stock tumor in the type of disease produced. The available evidence allows the statement that the numerical and structural chromosome alterations are important in the rise of new types of tumors, or that the development of tumors with a change in their properties takes place in close association with genotypic changes resulting in the rise of tumor sublines. There is particular interest in the rise of subline D because it arose in one of the rats that received intraperitoneal injection of refrigerated tumor cells of subline C. Though it may be insufficient to conclude that the refrigeration of tumor cells directly caused the chromosomal alteration leading to the origin of D, it is still important to note that subline D, characterized by the presence of 4 large V chromosomes, was derived from subline C of 3 V-type through inoculation of tumor cells subjected to freezing. In connection with the rise of tumors and their progression toward the change in type of the disease, it is a question whether or not the stemline chromosomes change during the development of serial transfers of tumors following their origin from the ordinary tissue cells. Evidence has been offered that the permanent changes of the stemline ideogram as well as its genotype cannot be induced by the drastic application of some chemicals. Some of the stem cells remain alive unaffected by chemicals and form the primary source of renewed malignant growth with the characteristic stemline chromosomes (*7*, *20*, *35*, *36*). However, the available data collected during the last several years indicate that the constancy of the stem line, while it is very pronounced, is not necessarily permanent, and that the ascites tumors during serial transfers have undergone numerical and structural changes of chromosomes. Several items of evidence supporting this statement have been presented by Hauschka (*29*) and Levan (*37*) in mouse ascites tumors. Recently, Makino (*9*, *38*), in MTK-tumor IV, and Tonomura and Sasaki

(*33*), in MTK-sarcomas II and III, have found, after a comparison of the stemline ideograms between early and recent transfer generations, that transitions have occurred during serial transfers in the stemline chromosomes with changes in type of disease in these tumors. The evidence presented in the present paper for the sublines of the Yoshida sarcoma furnishes fine examples strongly supporting this view. Especially the rise of subline D is interesting, since it is connected with such an experimental procedure as refrigeration. It seems probable that, under certain conditions, transition will occur in the genotype of the tumor with visible changes of the stemline chromosomes, and such changes will produce the shifts in the property of the tumor resulting in the rise of sublines. In this connection, Levan (*37*) has argued that the progression of tumors toward increased malignancy is connected with, and caused by, gradual genotypic changes.

In conclusion, following the chromosomal analysis, an important comment should be given here on the question concerning the origin of sublines A and C. Hamazaki *et al.* (*10, 11*) describe subline A as produced by means of intraperitoneal injections of the filtrate of the Yoshida sarcoma, which was prepared with the use of a Seitz-EK apparatus, and that subline C was induced by feeding the Yoshida sarcoma to nontumorous rats. These statements seem to indicate that each of the 2 sublines was induced by a cell-free agent. As mentioned in the foregoing, the 4 sublines A, B, C, and D are closely akin to each other in respect to their chromosomal relationship, and, further, they have a close relation to the original stock tumor in their chromosome constitutions. The cytological evidence has revealed that the 4 sublines are surely derivatives from the original stock tumor, all being cellular descendants from the latter. It is then evident that sublines A and C are exclusively of cellular origin, contrary to the view of cell-free origin as argued by Hamazaki *et al.* (*10, 11*). Here, serious doubt arises regarding the technical procedures by which they produced sublines A and C. Very probably, subline A was produced by the injection of the filtrate that contained a few tumor cells by unavoidable mistake in course of filtration, while subline C was caused by tumor cells that infiltrated through a wound (or wounds) on the epithelium of the alimentary tract when the tumor was fed to rats.

Summary

A comparative ideogram study of the Yoshida sarcoma and its 4 subline derivatives, with the use of an improved technique, is presented. The early view expressed by the senior author on the chromosomal relationship between the Yoshida sarcoma and the rat was revised on the basis of the study of new material.

The ideogram analysis carried out in special reference to the rise and distinction of sublines has established 4 different sublines in the Yoshida sarcoma. It has been shown that the 4 sublines are surely derivatives from the original stock tumor, all being cellular descendants from the latter. Each subline is characterized by its specific stemline ideogram,

which is clearly distinguishable from the others by the presence of 2, 3, and 4 V-shaped chromosomes of prominently large size, as well as from the stock tumor which is characterized by one such V chromosome. In addition to such gross distinction as marked by the large V chromosomes, there are many other differences between the 4 sublines in the structural features of the chromosomes other than the large V's and in the chromosome-number mode. It has then been stated that the numerical and structural chromosome alterations are important in the rise of new types of tumors, and that the development of tumors with a change in the type of disease takes place in close association with genotypic changes in stem cells and results in the rise of sublines. It is of particular importance to see that subline D, which is characterized by the presence of 4 V chromosomes, was derived from subline C of 3 V-type through the inoculation of their tumor cells subjected to freezing.

The available evidence shows that the constancy of the stem line, while it is very pronounced, is not necessarily absolute, and that the ascites tumors have undergone numerical and structural changes of chromosomes during serial transfers.

Addendum

After this article was completed and sent to press, T. H. Yosida reported before the 28th Annual Meeting of the Zoological Society of Japan, on August 30, 1957, the occurrence of a new subline of the Yoshida sarcoma which is clearly distinguishable from the stock tumor by the presence of a big J-shaped chromosome in addition to large two V's. It was stated that this subline was derived during serial transfers from a subline that was characterized by two large V chromosomes.

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PLATES

451329—58——4

PLATE 42

FIGURES 1 to 3.—Serial alignments of somatic chromosomes of white rats into supposed pairs. Figure 1, from liver culture, figure 2, from lung culture, and figure 3, testis ($2n$, 42).

FIGURES 4 and 5.—Serial alignments of chromosomes of stock tumor of Yoshida sarcoma, in approximate order of size (42 chromosomes)—*cf.* figure 4 with figure 29.

FIGURES 6 to 10.—Serial alignments of chromosomes of subline A of the Yoshida sarcoma, in approximate order of size. Figures 6 and 7, 40 chromosomes; figures 8 to 10, 39 chromosomes.

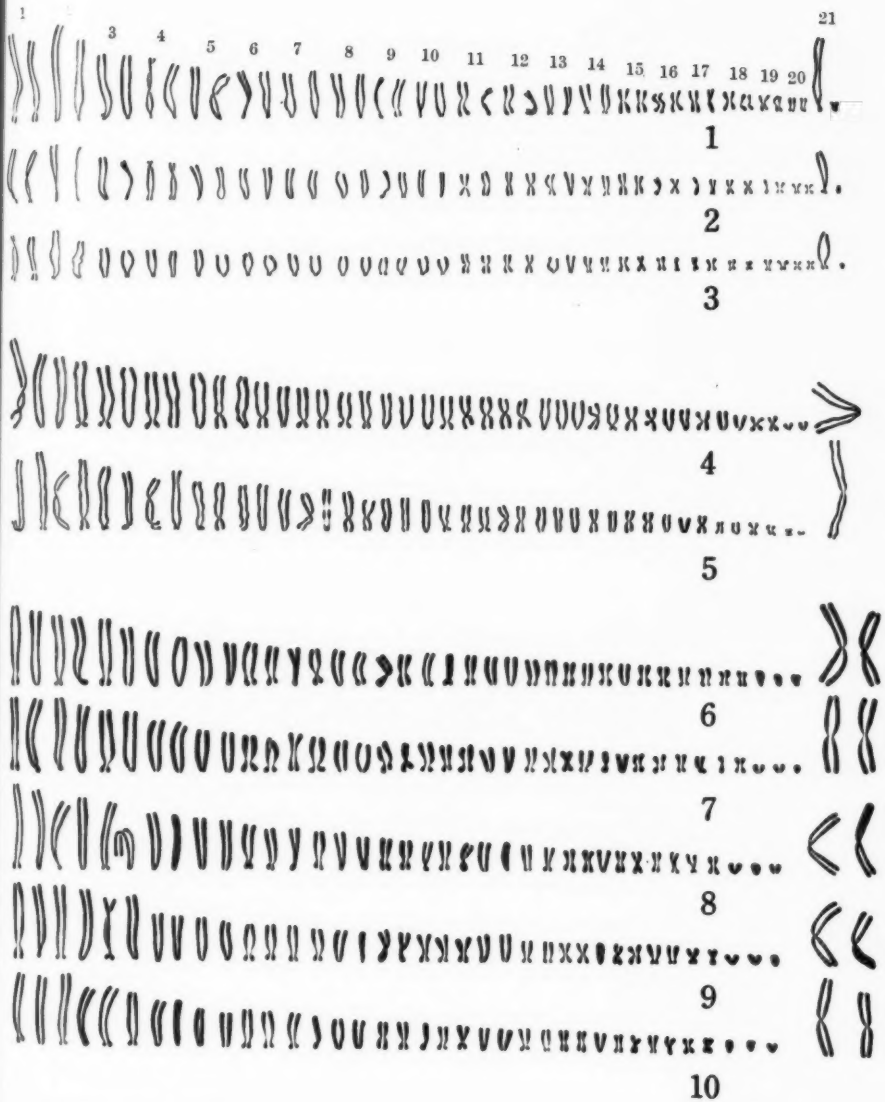

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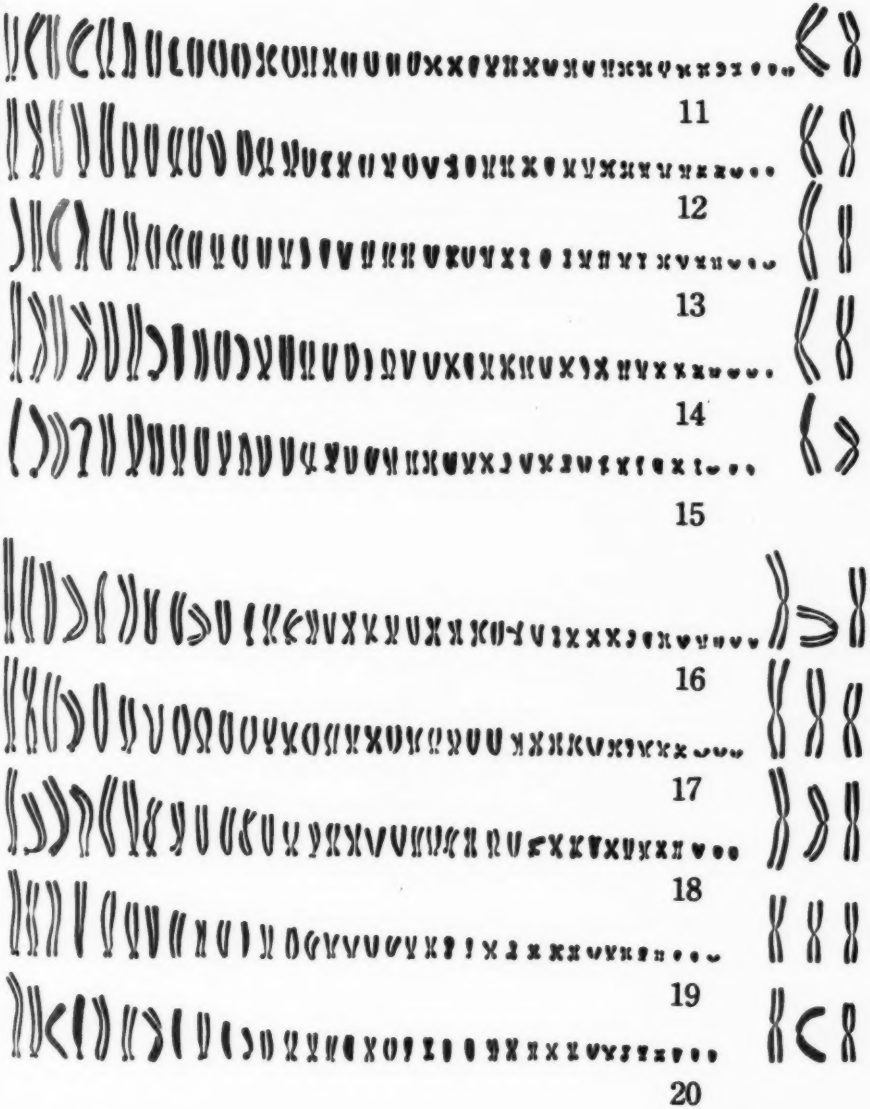
PLATE 43

FIGURES 11 to 15.—Serial alignments of chromosomes of subline B of the Yoshida sarcoma, in approximate order of size. Figure 11, 41 chromosomes; figures 12 to 14, 40 chromosomes; figure 15, 39 chromosomes.

FIGURES 16 to 20.—Serial alignments of chromosomes of subline C of the Yoshida sarcoma, in approximate order of size. Figure 16, 40 chromosomes; figures 17 and 18, 39 chromosomes; figures 19 and 20, 38 chromosomes.


11
12
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18
19
20

PLATE 44

FIGURES 21 to 25.—Serial alignments of chromosomes of subline D of the Yoshida sarcoma, in approximate order of size. Figure 21, 41 chromosomes; figures 22 and 23, 40 chromosomes; figure 24, 39 chromosomes; figure 25, 38 chromosomes.

FIGURES 26 and 27.—Serial alignments of chromosomes, from cells in the tissue-culture material of subline D of the Yoshida sarcoma, in approximate order of size (39 chromosomes in both).

21
22
23
24
25

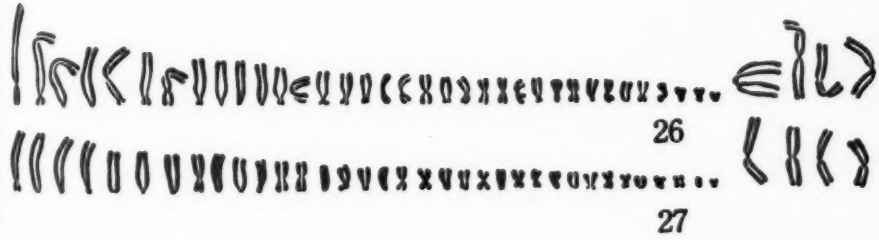
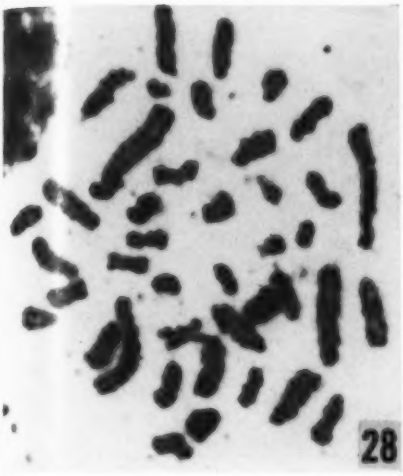

26
27

PLATE 45

FIGURES 28 to 31.—Photomicrographs of metaphase chromosomes. Figure 28, spermatogonial chromosomes of the rat (42 chromosomes), × 2,300. Figure 29, the Yoshida sarcoma, stock tumor (42 chromosomes), × 1,800. Characteristic large V-chromosome (*arrow*); large chromosome (*asterisk*) on this micrograph has a centromere closest to the end and therefore it is J-shaped. The chromosome of similar nature is also seen in figure 30 (*asterisk*). Figure 30, the Yoshida stock tumor with large V chromosome (*arrow*) and large J element (*asterisk*), × 2,000. Figure 31, the Yoshida sarcoma subline A (40 chromosomes), × 2,000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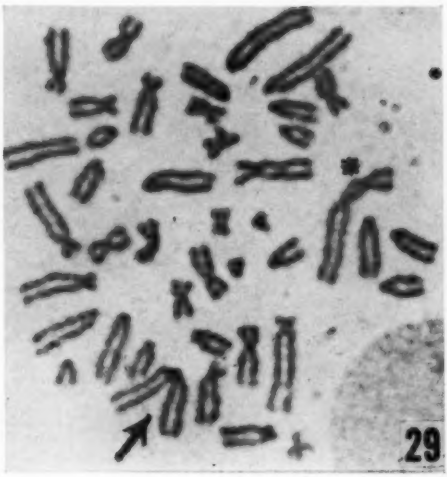
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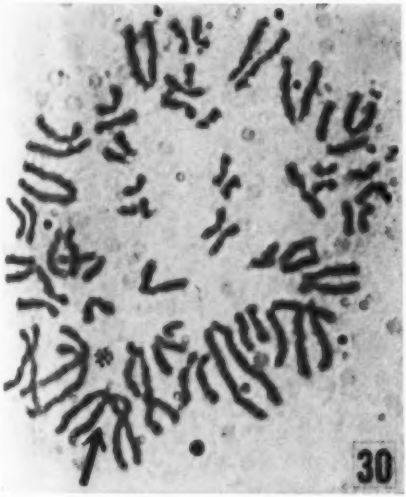
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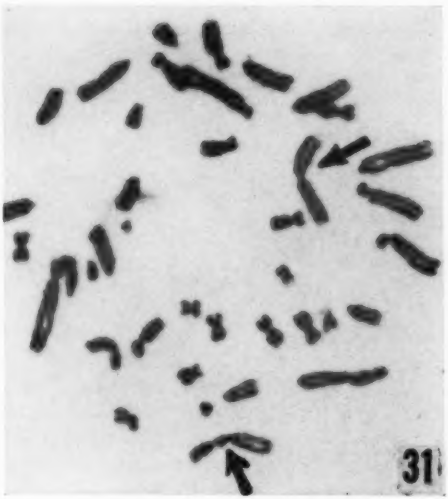
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PLATE 46

FIGURES 32 to 35. Photomicrographs of metaphase chromosomes. Figure 32, the Yoshida sarcoma, subline B (40 chromosomes), × 1,800. Figure 33, the Yoshida sarcoma, subline C (39 chromosomes), × 2,200. Figures 34 and 35, the Yoshida sarcoma, subline D (39 chromosomes). Figure 34, × 2,200. Figure 35, × 2,000 (*Arrows* indicate the large V-shaped chromosomes.)

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Further Evidence of Indirect Induction by X-radiation of Lymphocytic Neoplasms in Mice [1]

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Lymphocytic neoplasms arise in the thymus of certain strains of mice spontaneously, and also following treatment with X-radiation, carcinogenic hydrocarbons, or estrogens. Total thymectomy strikingly reduces the incidence and increases the mean age at death in the spontaneous disease of AK (*1*), AKR, and C58 strain mice (*2*), in the carcinogen-induced disease in DBA/2 mice (*3*), and in the X-ray-induced disease of C57BL and certain interstrain F_1 hybrids (*4–6*). In certain other strains of mice, notably the high-leukemic C3H/Fg subline of the C3H strain, lymphocytic neoplasms appear to arise at extra-thymic sites, and here thymectomy does not influence the incidence of these neoplasms (*7*).

The presence of an autologous or isologous thymic graft partially restores the potentiality of the host to develop lymphocytic neoplasms. In DBA/2 strain mice, thymectomy, followed by immediate autologous thymic grafting and application of a carcinogen, restored the host's potentiality to develop lymphocytic neoplasms (*3*). In other instances, under different conditions, employing C57BL/KA strain mice (*8*) and F_1 hybrids obtained by crossing C57BL by A/He strain (*6*) or C3H strain mice (*5*), thymectomy followed by a course of X-radiation and the implantation of a nonirradiated thymus from the susceptible parental strain restored this potentiality.

It is clear from the results using C57BL and F_1 hybrid mice that the site of origin of neoplasms is the thymic graft. The first evidence of a neoplasm is found by palpation of the graft, which enlarges progressively. There may occur a generalized lymphomatosis from this point of origin, or the neoplasm may remain localized even though attaining a large size.

Grafting of C57BL thymic tissue in C57BL thymectomized and irradiated mice was accomplished at from 1 hour to 8 days following the last of 4 exposures to X rays, in a study reported by Kaplan and Brown (*8*). Of the several lymphocytic neoplasms arising at the site of the nonirradiated thymic graft, one neoplasm arose from thymic tissue grafted as late as 8 days after the last X-ray exposure. These authors interpreted

[1] Received for publication June 20, 1957.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

their results to mean that lymphocytic neoplasms arose in thymic grafts which received no irradiation at any time, indicating that the initiation of leukemogenesis did not require exposure to X-radiation, but rather required an exposure to a postradiation influence.

The evidence that these results represented "the first definitive instance of the production of a malignant tumor in a tissue not exposed to the carcinogenic agent responsible for the neoplastic change" was indeed suggestive. Critical evidence, however, was not presented to establish this postulate, since in the experimental system used, differentiation could not be made between neoplasms arising from nonexposed cells of the thymic graft, or exposed cells of the host, which might populate the graft. Both the host and transplanted tissue were C57BL. A previous report from this laboratory established the possibility of repopulation of AKR thymic grafts by (C3H × AKR)F_1 host cells (*9*).

It is possible to differentiate between neoplasms arising from thymic cells not previously exposed to radiation and those arising from thymic grafts repopulated by host cells. Law and Potter (*6*) employed thymic tissue which was compatible, but genetically different from the host animal; C57BL thymic tissue was grafted into (C57 × A)F_1 hybrid hosts. The histocompatibility patterns of the resulting lymphocytic neoplasms (arising at the site of grafted thymic tissue) were determined by transplantation of neoplastic tissue into the parental strains, C57BL and A, and into the F_1 hybrid. The progenitor cells of these neoplasms arising in thymic grafts were found to be contributed either from the (C57BL × A)F_1 host (which received radiation), or, in a smaller number of cases, from the nonirradiated C57BL grafted tissue. The latter cases confirm in part the postulate of Kaplan and Brown of a postradiation influence. In this study (*6*), C57BL thymic tissue was grafted either at 1 day, or 7 days, following the last of 4 X-ray exposures.

Later, Kaplan and colleagues employed C57BL thymic grafts in (C57BL × C3H)F_1 hybrid mice (*5*). It was reported that their results, following transplantation of lymphocytic neoplasms arising from grafted thymic tissue, indicated that "the tumor cells behave genetically as if they were derived from cells of the implant."

In extensions of our work, thymic tissue from young donors, 1 to 10 days of age, were grafted subcutaneously in the right axillary region into the same interstrain hybrids used previously (C57BL × A)F_1. Subcutaneous implants were made at 14, 21, and 28 days after the last of 4 weekly exposures to X ray. Mice receiving 28-day thymic grafts were therefore approximately 3 months of age at the time of grafting. Methods employed and physical factors of radiation were identical to those described previously (*6*) (*see also* table 1).

It may be seen from table 1 that 9 neoplasms, to date, have arisen from thymic grafts transplanted to thymectomized (C57BL × A)F_1 hybrids which were irradiated 14 to 28 days prior to grafting. At necropsy a nodular mass, white and opaque, was found in the subcutaneous tissues of the right flank where the thymus had been implanted. Most neoplasms

TABLE 1.—*Lymphocytic neoplasms in thymic tissue grafted 14 to 28 days following fractionated X-radiation of thymectomized (B × A)F₁ mice**

Sex and no. of mouse	Donor thymus	Postradiation day of graft	Age animal sacrificed (*months*)	Transplantation behavior				Histocompatibility type
				1st transplant		2nd serial transplant		
				C57BL†	(B × A)F₁	C57BL	(B × A)F₁	
♀ 20226	C57BL	28	7. 0	61, N, N	64, 84, N, N, N	49, 49, N	46, 51, 51	C57BL
♀ 20229	C57BL	28	9. 0	N, N, N	N, N, 77	65, 58, 65	65, 55, 55	C57BL
♂322	C57BL	21	6. 5	N, N, N	36, 47, 47	N, N, N	32, 34, 35	(B × A)F₁
♂466	C57BL	14	6. 0	N, N, N	40, 45, 45, 50			(B × A)F₁
♂394	C57BL	28	8. 5	103, N, N	90, N, N	55, 55, 58, 55	N, 40, 53, 55	C57BL‡
♀ 333	C57BL	21	6. 0	38, 38, N, N	N, N, N			C57BL
♀ 1126	A/He	14	8. 5	No growth				
♀ 478	C57BL	14	11. 0	N, N, N, N	40, 40, 50, N			(B × A)F₁
♀ 386	C57BL	28	12. 0	N, N, N, N	55, N, N, N	No growth at 6 months		?

*Total-body irradiation of 225 r given weekly for 4 weeks. Mice were 30 days of age at first exposure. Physical factors were as follows: Two opposing tubes above and below mice were used, each 54 cm. from the mouse target; 200 kvp, 14 ma. (each tube); beam filtration, 0.25 mm. Cu and 0.55 mm. Al; 225 r was delivered in 1.75 minutes.

†Day at death from progressively growing neoplasm; N=no growth of neoplasm.

‡Carried serially through 5 transfers with progressive growth in both C57BL/KA and (C57BL × A)F₁ mice.

had attained the size of approximately 1 cm. in diameter at the time of sacrifice. Sacrifice of the animal, in most cases, was early enough so that little or no extension to other tissues had occurred. Two neoplasms appeared as early as 3 months after grafting of C57BL thymic tissue (*see* ♂ 466 and ♀ 333 in the table). All except one of the neoplasms grew progressively upon transfer, and the histocompatibility patterns of these neoplasms were determined as either C57BL or (C57BL × A)F_1, except in one instance. The latter, a lymphocytic neoplasm arising at 12 months in a 28-day postradiation graft (♀ 386), grew progressively and killed only 1 of 4 F_1 mice and has failed to grow to date in the 2nd transfer. Of the remainder, 4 definitely are of C57BL origin, arising from C57BL thymic tissue grafted at either 21 or 28 days after the last exposure of the animal to X rays. This indicates establishment of neoplasms from lineal descendants of thymic round cells, which have regenerated from the donor C57BL tissue and which had not themselves been exposed to direct X-radiation, as suggested by Kaplan and colleagues. The remaining 3 lymphocytic neoplasms grew progressively following transplantation and killed only (C57BL × A)F_1 mice, indicating their origin from host cells which repopulated the thymic graft. In all probability these neoplasms comprise descendants of cells which had received radiation.

Thymic tissue grafted into thymectomized, but nonirradiated, mice has not in a single instance given rise to a lymphocytic neoplasm. Fifty (C57BL × A)F_1 hybrids have been under observation for more than 17 months.

Within a 12-month observation period, 9 of 113 (C57BL × A)F_1 hybrids have developed lymphocytic neoplasms at the site of grafted thymic tissue. In contrast, but within a longer period of observation, 17 months, 12 of 38 (31.7%) and 12 of 48 (25%) of those C57BL hybrids bearing thymic tissue grafted either at 1 day, or at 7 days, postradiation, developed into neoplasms. The majority of these latter neoplasms (64%) revealed an F_1 (C57BL × A) pattern (*6*).

In the present series, all the neoplasms were lymphocytic, typical of those occurring in intact irradiated C57BL or interstrain F_1 hybrids, using C57BL as one parent. Upon occasion, type A and type B reticular neoplasms, as described by Dunn (*10*), have been encountered in grafted thymic tissue, particularly in our larger series comprising 1-day and 7-day postradiation grafts (*6*, *11*).

Summary

Thymectomized and irradiated (C57BL × A)F_1 hybrid mice receiving thymic tissue from C57BL donors as late as 28 days after irradiation develop lymphocytic neoplasms. These neoplastic cells arising at the site of thymic grafts have been shown by their genetic histocompatibility response to be derived either from nonirradiated cells of the thymic graft (C57BL) or from cells of the irradiated host (C57BL × A)F_1 which repopulate the graft. Thus, cells of the lymphoid series that are eventually transformed to the neoplastic state need not receive direct radiation. A

postradiation influence, which persists at least for 28 days in the (C57BL × A)F_1 mouse, has been detected.

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Effects of Certain Amino Acid Derivatives on Microorganisms and Sarcoma 180 [1, 2]

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A quantitative microbiological analysis of the free and combined amino acids in hydrolysates of mouse tumor tissue (sarcoma 180) and normal mouse tissue, *i.e.*, liver, spleen, heart, brain, kidney, muscle, and intestine revealed certain differences (*1*). These differences were used as a basis for selection of certain amino acid antimetabolites to interfere with the growth of sarcoma 180 in mice. It is the purpose of this report to describe results obtained in the treatment of mouse sarcoma 180 with amino acid analogues that were selected on the basis of the foregoing study.

The theory of selective toxicity based on quantitative differences between the tumor and host tissues in some essential metabolite or enzyme component has been given consideration (*2–5*).

The study of amino acid antagonists as chemotherapeutic agents against experimental tumors has shown some promise. Ethionine was shown to have inhibitory properties against adenocarcinoma 755 but not against sarcoma 180 (*6*). Two others, O-diazoacetyl-L-serine (azaserine) (*7*) and its analogue, 6-diazo-5-oxo-L-norleucine (DON) (*8*), though they are antibiotics recovered from cultures of *Streptomyces*, are also amino acid analogues. Both are effective against sarcoma 180, but do not destroy the growth potential of the tumor cells. Both have been shown to inhibit the conversion of *N*-formylglycinamide ribotide to 5-aminoimidazole ribotide in purine synthesis by a cell-free preparation from mouse plasma-cell neoplasm 70429 (*9*). In a pigeon liver system it has been shown that the reaction inhibited by azaserine is reversible with glutamine. Azaserine is therefore regarded as a glutamine-antagonist (*10*). DON is presumed to act similarly. The biological activity of the acid hydrazines of amino acids against bacteria was investigated by Roper and McIlwain (*11*). They studied γ-glutamyl hydrazine, its acetone derivative β-aspartyl hydrazine, and hydrazine itself for interference with glutamine utilization of the Richards strain of hemolytic streptococcus. They found β-aspartyl hydrazine to be the least active of the

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[2] This investigation was aided in part by research grant C-2479 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service and, in part, by the Midwest Research Institute.

compounds tested, 0.04 *M* was required to inhibit either *Streptococcus faecalis* or the Richards strain for more than 24 hours and the inhibition could be diminished by addition of 0.01 *M* asparagine or 0.0004 *M* glutamine.

We have found that 0.0037 *M* DL-β-aspartyl hydrazine [3] in presence of 0.0015 *M* L-aspartic acid completely inhibited growth of *Lactobacillus delbrueckii* for at least 72 hours and the inhibition was not reversed by 0.37 *M* L-aspartic acid, table 4.

Experimental data which led to the use of the aspartic acid analogue in the therapy of sarcoma 180 were the lower content of this amino acid in the tumor than in the normal tissues of the mouse and the presence of a greater concentration of aspartic acid in the nontumorous tissues of the tumor-bearing mice than those same tissue types in normal mice (*1*); also the role that aspartic acid contributes in the biosyntheses of purines and pyrimidines in tissues (*12*).

The data on the amino acid composition of sarcoma 180 and various normal mouse tissues showed aspartic acid, histidine, leucine, isoleucine, and valine to be lower in tumor-tissue protein (*1*). The DL-β-aspartyl hydrazine was studied for its effect on sarcoma 180 in mice alone and in combination with deoxypyridoxine. Deoxypyridoxine was used because it was known to depress the aspartic-glutamic transaminase activity of tissues (*5*) and the combination of both a substrate inhibitor, aspartyl hydrazine, and the transaminase inhibitor offered the possibility of augmenting each other.

Experimental

Methods.—Strain BALB/c mice were used as hosts for the sarcoma 180 tumor. They were implanted with a 2 mm.^{3} piece of fresh tissue, obtained from a week-old tumor. Implants were made subcutaneously in the right axillary region by the usual trocar method. Therapy was administered by daily intraperitoneal injections of the drug dissolved in 3 percent gelatin at the dosage indicated, beginning 72 hours after tumor implantation. Animals were allowed free access to Purina laboratory chow and water. Food and water consumption and animal weights were recorded at the time of each injection. At the conclusion of the experiment the animals were killed by cervical dislocation, and the tumors were removed and weighed.

The microbiological test organism used for determining biological activity was *Lactobacillus delbrueckii* NRRL-LD5 with the test medium of Stokes, Gunness, Dwyer, and Caswell (*13*).

Aspartic-glutamic acid transaminase values for the tissues were determined by the method of Tonhazy, White, and Umbreit (*14*). Ribonucleic acid and deoxyribonucleic acid were determined on the tissues by the orcinol reactions (*15*) and diphenylamine reaction (*16*), respectively. Tissue extracts were prepared according to Ceriotti (*17*).

[3] Prepared by Dr. L. H. Goodson and S. Brand.

Results

Tests for Biological Activity of Aspartic Acid Analogues

Among the aspartic acid analogues that were tested for their ability to antagonize the utilization of L-aspartic acid by *L. delbrueckii* were DL-β-aspartyl hydrazine, α-methylaspartic acid,[4] anti-hydroxyaspartic acid, DL-carbamylaspartic acid and the methyl ester of epoxysuccinic acid hydrazide. With the exception of α-methylaspartic acid all of these compounds were prepared in these laboratories by Dr. L. H. Goodson and S. Brand on a program supported by grant-in-aid C-1816 from the National Cancer Institute.

α-Methylaspartic acid and hydroxyaspartic acid both inhibited the growth of *L. delbrueckii* and the inhibition was reversed by addition of L-aspartic acid. In the presence of L-aspartic acid concentrations giving one-half maximum growth, α-methylaspartic acid showed inhibition beginning at 6:1 ratio, which was not complete until a 133:1 ratio had been reached. It was reversed by addition of L-aspartic acid at 0.05:1 ratio.

Hydroxyaspartic acid inhibited at a 100:1 ratio and was reversed by L-aspartic acid at a 3:1 ratio.

Both carbamylaspartic acid and methylepoxysuccinic hydrazide were utilized by the bacterium and showed no inhibition of aspartic acid utilization.

Inhibition of Aspartic-Glutamic Transaminase Activity in Normal and Tumor Mouse Tissues by Deoxypyridoxine

Shapiro, Shils, and Dietrich (*5*) reported the transaminase values of several normal tissues and tumor tissue, adenocarcinoma 755, in both deoxypyridoxine-treated and untreated mice. The tissues from treated animals all showed a depression of transaminase activity.

To establish whether deoxypyridoxine influenced the aspartic-glutamic acid transaminase activity of normal and sarcoma 180 tissue of mice, measurements of the content of these enzyme systems were made on liver and tumor tissues of untreated animals and animals that had been treated with deoxypyridoxine (table 1). The tissues were removed from the animals and placed immediately on cracked ice. Samples were carefully

TABLE 1.—*Effect of deoxypyridoxine on the aspartic-glutamic transaminase activity of mouse liver and tumor tissues**

Tissue	No. samples	Avg. Q_{10}^{T} value
Normal mouse liver	24	318
Mouse liver, treated	18	170
Sarcoma 180 tissue	18	84
Sarcoma 180 tissue, treated	18	30

*150 mg./kg. of deoxypyridoxine I. P. daily for 9 days.

[4] Obtained through the courtesy of Dr. W. W. Umbreit, of Merck Institute for Therapeutic Research.

weighed, homogenized in phosphate buffer, and analyzed according to the method of Tonhazy, White, and Umbreit (*14*).

The data demonstrate that the drug does indeed depress the transaminase activity, 46.5 percent in the case of liver and 64.3 percent in the case of the tumor tissue. This is in accord with the idea that the lower the initial enzyme concentration of a tissue the greater will be the inhibition by an antagonist of the enzyme. Pyridoxal phosphate functions as a coenzyme of aspartic-glutamic transaminase (*18*, *19*). We have, however, found no clear evidence of a relationship between the degree of transaminase inhibition and tumor inhibition.

Effect of DL-β-Aspartyl Hydrazine Alone and in Combination with Deoxypyridoxine on Sarcoma 180 in Mice

In view of the rather strong antagonism exerted by aspartyl hydrazine against the utilization of aspartic acid by *L. delbrueckii*, it was tested against sarcoma 180 in mice. On the theory that a combination of an enzyme inhibitor combined with a substrate antimetabolite would enhance the effectiveness against the tumor, deoxypyridoxine was used along with aspartyl hydrazine. The acute toxic dose of the aspartyl hydrazine determined for mice was approximately 1,500 mg. per kg. of body weight, that for deoxypyridoxine was 300 mg. per kg. of body weight.

Male mice weighing about 25 gm. were implanted with sarcoma 180 tissue as described under "Methods." Seventy-two hours after implantation, daily intraperitoneal injections of the drug or drugs were begun and continued daily for 9 successive injections. The controls received injections of the 3 percent gelatin solution that was used as a solvent for the drugs. On the morning of the 10th day after therapy was begun, the animals were sacrificed and the tumors were removed and weighed. If analyses were to be made on other tissues they were also removed and placed on ice until samples had been taken for analysis. The data in table 2 show the effects of these two drugs, singly and in combination, on the growth of sarcoma 180 in mice.

The inhibition of tumor growth under these experimental conditions with aspartyl hydrazine alone was about 40 percent. When deoxypyridoxine was used in combination with the aspartyl hydrazine, the inhibition was 70 to 80 percent. Thus the 2 compounds supplement each other and the effects were additive. The results of 6 separate experiments in which these 2 compounds were used singly and in combination are shown.

Except for the differences in size there were no gross differences in the tumors from the treated animals when compared with the untreated tumors. In spite of the fact that tumor growth was held in partial check during the period of treatment, the weight losses of the treated animals were, in most cases, greater than in the untreated controls. The weight loss of the treated animals was most severe during the first 5 days of therapy. Treated animals consumed less than one half the food as compared to tumor-bearing control animals; 1.5 gm. as compared to 3.5 to 4.0 gm. per day per animal.

TABLE 2.—*Effect of aspartyl hydrazine and deoxypyridoxine on the growth of sarcoma 180 in mice**

Expt.	No. of mice surviving	Dosage *(mg./kg.)*	Avg. tumor weight *(gm.)*	% Reduction in tumor weight	Wt. losses of animals	
					Gm./mouse	% Initial wt.
1. Control	10/10	0	1. 301 ± 0. 246	—	4. 65	18. 5
DP	9/10	150	0. 732 ± 0. 256	43. 5	4. 15	17. 4
AH	10/10	150	0. 618 ± 0. 202	53. 0	5. 90	23. 7
2. Control	9/9	0	1. 182 ± 0. 228	—	4. 3	16. 0
DP	10/10	150	0. 819 ± 0. 131	31. 5	2. 6	9. 5
DP + AH	8/10	150 + 150	0. 347 ± 0. 101	70. 5	4. 8	17. 6
3. Control	10/10	0	1. 113 ± 0. 167	—	4. 1	16. 3
DP	9/10	150	0. 680 ± 0. 249	39. 0	1. 3	5. 2
DP + AH	10/10	150 + 150	0. 331 ± 0. 121	71. 3	4. 5	17. 4
4. Control	5/5	0	1. 320 ± 0. 500	—	2. 4	10. 0
DP + AH	5/5	150 + 150	0. 240 ± 0. 120	82. 0	5. 7	22. 3
5. Control	6/6	0	1. 053 ± 0. 269	—	4. 94	21. 9
AH	6/6	150	0. 625 ± 0. 311	40. 8	4. 86	22. 3
DP + AH	6/6	150 + 150	0. 253 ± 0. 153	76. 3	4. 20	18. 4
6. Control	10/10	0	0. 958 ± 0. 145	—	3. 6	14. 2
AH	7/10	150	0. 578 ± 0. 191	39. 6	6. 8	26. 7
DP + AH	9/10	150 + 150	0. 285 ± 0. 140	70. 5	6. 9	27. 0

*AH=DL-aspartyl hydrazine; DP=4-deoxypyridoxine hydrochloride. Treatment was begun 72 hours after tumor implantation with a dosage of 150 mg. per kg. of each drug daily for 9 successive days. All animals were sacrificed 13 days after implantation.

Effect of Caloric Restriction on Tumor Growth

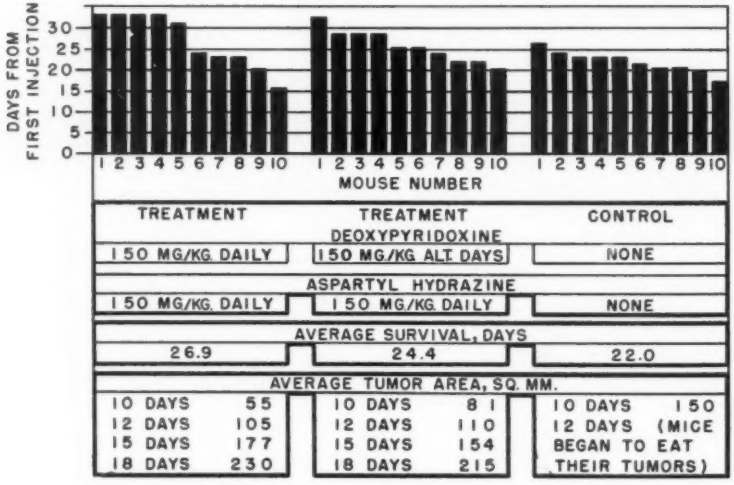
Caloric restriction is known to inhibit the growth of transplanted tumors (*20*). Experiments to determine whether the tumor inhibition was due to a restriction of food intake by the animal when they received the drugs or to a partially selective effect of the drugs on the proliferation of tumor tissue were carried out and the data are shown in table 3.

TABLE 3.—*Effect of food restriction on the growth of sarcoma 180 in mice**

Expt.	Group	No. of mice surviving	Avg. tumor weight *(gm.)*	Percent inhibition of tumor	Weight loss of animals	
					Gm. per mouse	Percent of original weight
1	a	10/10	—	—	9. 1	38. 2
	b	9/10	0. 862 ± 0. 149	9. 1	8. 0	30. 5
	c	9/10	0. 948 ± 0. 233	—	5. 4	21. 0
2	a	10/10	—	—	7. 2	28. 9
	b	10/10	0. 630 ± 0. 112	36. 1	7. 4	28. 8
	c	10/10	0. 986 ± 0. 327	—	3. 9	14. 6

*Group (a) Normal animals—2.0 gm. of food daily for 10 days.
Group (b) Tumor-bearing animals—2.0 gm. of food daily for 10 days.
Group (c) Tumor-bearing animals—free access to food and water.

The experiments were set up as follows: 1) 10 animals were implanted and allowed free access to food and water; 2) 10 were implanted and allowed a 2.0 gm. portion each day of pulverized Purina laboratory chow and free access to water; 3) 10 normal animals were included and treated as for group (b). They were kept on these experimental conditions for



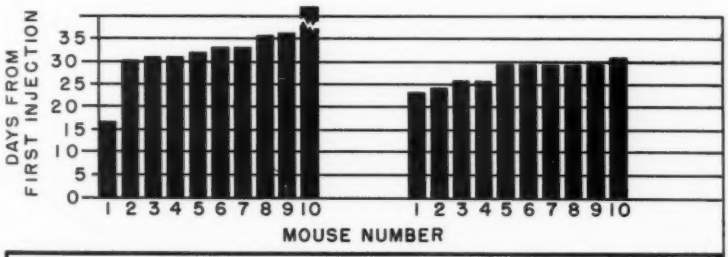
TEXT-FIGURE 1.—Survival time of sarcoma-180-bearing mice that were treated with deoxypyridoxine and aspartyl hydrazine for 10 successive days.

the same length of time as were those which had been subjected to drug treatment. The animals given the restricted diet were caged individually and the food was placed in a clean petri-dish lid each day. All of the food was consumed. The animals adjusted to this restriction in caloric intake and 2 mice without tumors were maintained on this food ration for 90 days before they were sacrificed.

The weight loss of the animals under these experimental conditions was more severe than for any of the animals given aspartyl hydrazine-deoxypyridoxine therapy. Inhibition of tumor growth was less also. It is doubtful that the inhibitions of tumor growth observed with the combination therapy were caused by starvation alone. This will be more evident when the results from the survival experiments are considered (*see* text-fig. 3).

Several experiments have been carried out to determine the effect of the treatment of tumor-bearing mice on their survival after tumor implantation. In one case the animals were treated with aspartyl hydrazine and deoxypyridoxine for 9 successive days and the treatment was then discontinued (text-fig. 1). In the other, the treatment was given daily, except on weekends, for as long as the mice survived (text-fig. 2). The area of the tumors was measured several times during the course of the experiment.

The bar graph in text-figure 1 shows the time of survival of each mouse in days. Two treated and one control group of 10 animals each were



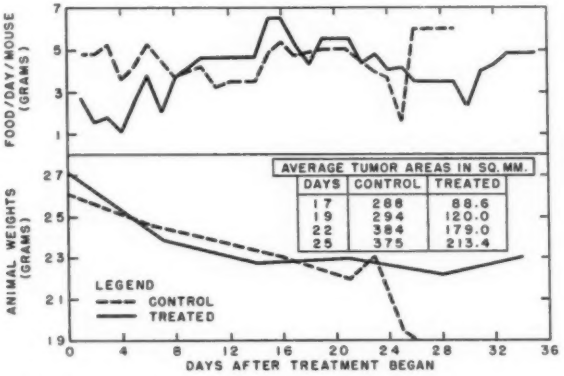
TREATMENT		CONTROL		
DEOXYPYRIDOXINE				
150 MG/KG. DAILY		NONE		
ASPARTYL HYDRAZINE				
150 MG/KG. DAILY		NONE		
AVERAGE SURVIVAL, DAYS				
31		28		
AVERAGE TUMOR AREA, SQ. MM.				TREATED / CONTROL
17 DAYS	88.6	17 DAYS	288	.30
19 DAYS	120.0	19 DAYS	294	.41
22 DAYS	179.0	22 DAYS	384	.47
25 DAYS	213.4	25 DAYS	375	.57

TEXT-FIGURE 2.—Survival time of sarcoma-180-bearing mice under continuous treatment.

included. At the end of 10 days there was a considerable difference in average tumor area between the treated and untreated animals. However, when treatment was discontinued rapid growth of the tumor ensued. In the treated group that received daily injections of both drugs, 5 of the 10 animals lived beyond 30 days. The control animals began to gnaw at their tumors so severely after the 10th day that area measurements were not of significance.

In the second experiment the animals received daily injections of both drugs throughout the course of the experiment. Two groups of 10 animals each were included, one control and one treated group. Mouse #10 in the treated group did not develop a tumor but was allowed to remain as a normal control receiving daily injections of the drug. There was no greater difference in length of survival between the control and treated groups of the animals on continuous treatment than in those that were treated for only 10 days. The data showing survival of the animals are given in text-figure 2. At the bottom of the graph are the measurements of tumor areas at 4 different periods during the experiment. Up to 17 days the tumor growth of the treated group was under rather good control, being less than one third that of the control group. However, the ratio of the average tumor area, treated animal tumors/control animal tumors, began to approach 1 at an accelerated rate: at 17 days, 0.31; at 19 days, 0.41; at 22 days, 0.47; and at 25 days, 0.57. From text-figure 3 it can be seen that the food consumption of the treated animals was decreased rather sharply until the 7th day of treatment. From that time on the treated and untreated animals consumed food at approximately the same rate. Also from text-figure 3, it can be noted that the body weights of the animals in each group remained nearly the same throughout. The divergence near the end of the experiment is caused by averages that are based on fewer animals.

It is difficult to dissociate the effect of the drug treatment on tumor growth completely from the effect of starvation. However the data in



TEXT-FIGURE 3.—Survival experiment: 150 mg. per kg. daily of aspartyl hydrazine and 150 mg. per kg. daily of deoxypyridoxine.

table 3 show much greater weight losses with less tumor inhibition than is obtained in the treated animals. It appears that a lowered response of the tumor to aspartyl hydrazine and deoxypyridoxine occurs rather rapidly. The mechanism of this phenomenon is unknown but the following results obtained with *L. delbrueckii* offer a possible explanation.

Development of Resistance by L. delbrueckii *to Inhibition of Growth by DL-β-Aspartyl Hydrazine*

The fact that DL-β-aspartyl hydrazine inhibits the utilization of L-aspartic acid by *L. delbrueckii* NRRL-LD5 is demonstrated in table 4. Virtually complete growth inhibition of this organism resulted with 0.0037 *M* DL-β-aspartyl hydrazine in the presence of 0.0015 *M* L-aspartic acid and the inhibition was not reversed by as much as 0.37 *M* L-aspartic acid. Continued transfer of *L. delbrueckii* in media containing L-aspartic acid with inhibitive concentrations of aspartyl hydrazine resulted in the emergence of a population of cells that became resistant to progressively higher concentrations of aspartyl hydrazine and eventually the culture was able to utilize aspartyl hydrazine as a substitute for its aspartic acid requirement (*see* table 5).

TABLE 4.—*Inhibition of* L-*aspartic acid utilization by* L. delbrueckii *with* DL-β-*aspartyl hydrazine* (DL-*AH*)*

γ L-Aspartic acid per tube		200 γ L-Aspartic acid per tube + DL-AH		500 γ DL-AH + L-aspartic acid per tube	
		γ DL-*AH*		γ L-*aspartic acid*	
0	1. 10 ml.	0	8. 70 ml.	0	1. 50 ml.
50	2. 50	50	5. 95	300	1. 40
100	6. 20	200	4. 40	900	1. 40
200	7. 70	500	1. 50	1, 800	1. 60
300	7. 80			5, 000	2. 50
				10, 000	2. 50
				30, 000	2. 15
				50, 000	1. 90

*Ml. of 0.1 *N* acid per 10 ml. of medium.

Some data showing the development of a resistance to DL-aspartyl hydrazine by *L. delbrueckii* is shown in table 5. In this test 200 γ of aspartic acid was added to 10 ml. of basal medium of Stokes *et al.* (*13*) along with quantities of aspartyl hydrazine from 200 γ to 1,000 γ. When 500 γ of aspartyl hydrazine was added to 200 γ of L-aspartic acid growth was effectively inhibited—inhibition which was not reversed by as much as 50,000 γ of L-aspartic acid (*see* table 4). Two tubes with each concentration of inhibitor were used. The first tube of the series was inoculated with 1 drop of washed bacterial suspension prepared as for amino acid assay. After 24 hours' incubation, 4 drops were used to transfer to the next tube in the series containing the inhibitor and aspartic acid. The cultures were incubated for 72 hours and titrated with 0.1 *N* NaOH.

From these results it appears that *L. delbrueckii* does develop a resistance to aspartyl hydrazine upon continued transfer in a medium con-

TABLE 5.—*Growth of* L. delbrueckii *in media containing* DL-*aspartyl hydrazine**

(a) γ L-Aspartic acid per 10 ml.		(b) 200 γ L-Aspartic acid per 10 ml. + varying concentrations of DL-aspartyl hydrazine DL-AH		(c) γ DL-Aspartyl hydrazine per 10 ml. (no aspartic acid) DL-AH	
0	0. 85	0γ	7. 45	500γ	6. 20
100	4. 90	200	5. 30	500	6. 25
200	7. 45	200	5. 10	500	5. 65
300	8. 30	300	3. 40	500	4. 85
500	8. 60	300	2. 55	500	5. 40
800	8. 55	500	1. 90	800	7. 00
		500	5. 65	1, 000	6. 40
		800	7. 25	1, 500	6. 45
		800	6. 40		
		1, 000	7. 90		
		1, 000	7. 00		
		1, 500	6. 50		
		1, 500	5. 90		
		2, 000	5. 70		
		2, 000	6. 75		

*Values in ml. of 0.1 *N* acid per 10 ml. of medium (72 hours' incubation at 37° C.).

taining the inhibitor. The first evidence of resistance developed on the 8th transfer, and in the next 2 transfers twice the concentration of inhibitor normally needed for complete inhibition of growth was without effect.

It has been found that cells transferred from cultures that show growth in the presence of DL-aspartyl hydrazine are able to utilize DL-aspartyl hydrazine to supply their aspartic acid requirements. Growth does not occur in the absence of aspartic acid or aspartyl hydrazine.

Transfer was made from a 24-hour culture of *L. delbrueckii* in media containing 800γ per tube of aspartyl hydrazine and 200γ of L-aspartic acid (column b) to media containing 500γ per tube of DL-aspartyl hydrazine and no aspartic acid (column c). Successive transfers, of 4 drops of culture at 24-hour intervals in this medium through 8 cultures, demonstrated the ability of the organism to utilize the aspartyl hydrazine as partial replacement for aspartic acid (table 5). Acid formation did not exceed that in the presence of 200γ of aspartic acid even where aspartyl hydrazine equivalent to 1,000γ of aspartic acid was present.

The resistance of the tumor in mice to growth inhibition by DL-aspartyl hydrazine and deoxypyridoxine (text-fig. 3) may develop similarly. Some evidence has been obtained that in the case of *L. delbrueckii* this development of resistance consists of removal of nitrogen as ammonia from the hydrazine group.

Treatment of Sarcoma 180 in Mice by Combinations of Deoxypyridoxine and Aspartyl Hydrazine with Other Tumor Inhibitors and Potential Tumor Inhibitors

In view of the considerable amount of information now available on pathways of biosynthesis of purines and pyrimidines (*12*) one may attempt a more rational use of amino acid antagonists and other inhibitors.

Aspartic acid is required as a first component in the presently proposed pathway of pyrimidine synthesis. Both L-aspartic acid and L-glutamine contribute to the synthesis of the purine skeleton. In addition, azaserine and folic acid analogues are known to interrupt steps in the biosynthesis of purines. In pigeon liver preparations azaserine presumably functions as an antimetabolite of glutamine since its effect can be reversed by large concentrations of L-glutamine (*10*).

In the experiment (table 6) both DL-aspartyl hydrazine and L-γ-glutamyl hydrazine [5] have been employed. Deoxypyridoxine was used to impede the formation of either aspartic or glutamic acid by the transamination reaction.

Of special note is the lack of effect on tumor growth by the L-glutamyl hydrazine alone. When combined with deoxypyridoxine, however, the tumor inhibition was equal to or perhaps stronger than that resulting from equivalent dosages of aspartyl hydrazine and deoxypyridoxine. The hydrazines of both L-aspartic and L-glutamic acid appear, when combined with deoxypyridoxine, to have an inhibitive effect on the growth of sarcoma 180 in mice. L-Glutamyl hydrazine was without effect on glutamic acid utilization by *Lactobacillus arabinosus* when used at ratios as high as 10 to 1 of L-glutamic acid. The effect of a number of other different combinations of drugs on the growth of sarcoma 180 is shown in table 6.

A third hydrazine derivative of interest was DL-histidine hydrazine monoacetate.[6] When tested for inhibition of histidine utilization by *Streptococcus faecalis* R. NRRL at ratio of 30 parts of the antagonist to 1 of histidine, there was no inhibitory effect on growth. When tested against sarcoma 180 in mice at the 250 mg. per kg. level there was no effect on tumor growth though there was a decrease in food intake and a considerable weight loss (20%) among the animals. When it was used with deoxypyridoxine, each drug given at a rate of 150 mg. per kg., the tumor growth inhibition was 85 percent. The histidine compound strongly resembled the L-glutamyl hydrazine in its effect on sarcoma 180.

When amethopterin, azaserine, or 6-mercaptopurine are added to a combination of aspartyl hydrazine and deoxypyridoxine there is an enhancement of tumor inhibition above that which is obtained when the combination or the single drug is used alone. Azaserine (administered separately, expts. 4 and 5) has proved most effective and has resulted in inhibitions, under these experimental conditions, in the region of 95 percent. In experiment 4, table 6, azaserine was used to supplement a triple combination of glutamyl hydrazine, aspartyl hydrazine, and deoxypyridoxine. In this case the dosage of all of the drugs was reduced. Though several tumors in these groups were unusually small, 5 to 10 mg., they resumed growth when the treatment was withdrawn. Amethopterin was not quite as effective as azaserine, experiment 7, table 6, and when the dosage was raised above 1.5 mg. per kg. a severe loss of animals

[5] Purchased from the Mann Research Laboratories, New York.
[6] Prepared by Dr. L. H. Goodson and S. Brand.

TABLE 6.—*Effect of various combinations of agents on the growth of sarcoma 180 in mice**

Expt.	Treatment	Dosage (*mg./kg.*)	No. of survivors	Avg. tumor weight (*gm.*)	Percent inhibition	Wt. loss in animals	
						Gm./mouse	% Orig. weight
1	O	—	10/10	1. 012 ± 0. 266	—	4. 0	14. 0
	GH	150	10/10	0. 946 ± 0. 486	6. 5	5. 3	18. 9
	GH; DP	150; 150	5/10	0. 158 ± 0. 051	85. 0	5. 1	18. 3
2	O	—	10/10	1. 138 ± 0. 331	—	2. 7	10. 1
	GH; AH; DP	25; 50; 150	9/10	0. 340 ± 0. 120	70. 1	2. 9	11. 1
	GH; AH; DP	37.5; 75; 150	9/10	0. 244 ± 0. 087	79. 0	3. 9	15. 4
3	O	—	10/10	0. 924 ± 0. 317	—	2. 9	11. 3
	GH; AH; DP	50; 75; 100†	10/10	0. 153 ± 0. 082	83. 5	5. 3	19. 0
	GH; AH; DP	50; 75; 100	5/10	0. 156 ± 0. 095	83. 0	6. 0	22. 2
4	O	—	10/10	0. 941 ± 0. 281	—	2. 5	9. 5
	GH; AH; DP	50; 75; 50	9/10	0. 265 ± 0. 162	73. 0	7. 5	27. 7
	GH; AH; DP; AZ	50; 75; 50; 2.0‡	7/10	0. 048 ± 0. 028	95. 0	7. 5	29. 7
5	O	—	10/10	1. 268 ± 0. 326	—	3. 9	14. 5
	AH; DP	150; 150	4/5	0. 489 ± 0. 146	61. 2	5. 0	21. 0
	AZ	5	5/5	0. 318 ± 0. 283	75. 0	3. 6	14. 5
	AH; DP; AZ	150; 150; 5	7/10	0. 064 ± 0. 032	95. 0	5. 9	23. 4
6	O	—	10/10	1. 030 ± 0. 203	—	3. 5	14. 2
	AH; DP	150; 150	5/5	0. 572 ± 0. 105	44. 5	6. 4	25. 7
	6-MP	5	5/5	0. 562 ± 0. 105	45. 5	2. 9	11. 9
	AH; DP; 6-MP	150; 150; 5	8/10	0. 169 ± 0. 077	83. 5	6. 8	25. 5
7	O	—	10/10	1. 082 ± 0. 189	—	5. 5	21. 0
	AM	1.5	10/10	0. 805 ± 0. 234	25. 6	0. 4	1. 6
	AM	3.0	10/10	0. 433 ± 0. 212	60. 0	1. 5	5. 8
	AH; DP	150; 150	9/10	0. 433 ± 0. 197	60. 0	4. 7	18. 0
	AH; DP; AM	150; 150; 1.5	7/10	0. 307 ± 0. 142	71. 6	4. 2	15. 9

*GH=L-glutamyl hydrazine; AH=DL-aspartyl hydrazine; DP=deoxypyridoxine; AZ=azaserine; AM=amethopterin; 6-MP=6-mercaptopurine.
†Fifty mg./kg. at 8 a.m. and 50 mg. at 5 p.m. daily.
‡Three drugs given in a.m., azaserine in p.m.

resulted. 6-Mercaptopurine, experiment 6, seemed intermediate in its effect when combined with aspartyl hydrazine and deoxypyridoxine. We have not observed inhibitions in excess of those reported with this combination.

The functioning of L-glutamine in two of the steps of inosinic acid synthesis in pigeon liver preparations led to the trial of L-glutamyl hydrazine as an inhibitor of sarcoma 180. In line with the idea of sequential blocking (*21*) of steps in the synthesis of purines the combined effects on sarcoma 180 of aspartyl and glutamyl hydrazines, deoxypyridoxine, and azaserine were determined. In this case the dosage level of the drugs was reduced and tested at several different levels. Experiments 2 and 3 of table 6 were experiments with the triple drug combination and experiment 5 is the triple combination with added azaserine at a rate of 2.0 mg. per kg. The dosage of the combined glutamyl and aspartyl hydrazine was adjusted to not exceed the amount of aspartyl hydrazine that had been used routinely, *i.e.*, 150 mg. per kg. The DL-aspartyl hydrazine was assumed to be active only in proportion to its content of the L-isomer. The deoxypyridoxine dosage was reduced to 100 mg. per kg. and in experiment 5 this was administered in 2 daily equal doses 8 hours apart. This procedure was based on the finding that the acute toxic dose was much less than the chronic toxic dose. Though the tumor inhibition was about the same with the two methods of administration, there was a lower animal mortality and less loss of weight among the survivors when the daily dosage of deoxypyridoxine was given in 2 daily doses rather than a single injection. When 2.0 mg. per kg. of azaserine was used with the triple drug therapy the deoxypyridoxine was reduced to 50 mg. per kg.

Effect of Treatment of Sarcoma 180 with Aspartyl Hydrazine, Deoxypyridoxine, and the Combination of Both Drugs on the RNA and DNA Content of the Tumor and Liver Tissues

Since the presumption was that this combination of drugs would interfere with the incorporation of aspartic acid in the biosynthesis of purines and pyrimidines and finally the nucleic acids, an analysis was made of the tissues to determine whether data would support this belief. Data from the analysis of the liver and tumor of animals that has been treated with either drug singly and the two in combination are given in the following tables. Tables 7 and 8 show typical results of experiments in which each drug was used alone and in combination.

Table 7 shows the values for RNA and DNA found in the liver and tumor tissue of mice. They are the average values of all the experiments, 18 in the control group and 13 in the group which received aspartyl hydrazine and deoxypyridoxine in combination, and 4 in each of the groups receiving the single drug. The value in each experiment was obtained by analysis of a composite tissue sample which was from 10 mice. Values are given as percent based on lipide-free dry tissue.

Judged by the amount of variation between samples, the most reliable values were obtained for the 2 groups with the largest numbers of experi-

TABLE 7.—*Nucleic acid content of mouse liver and tumor tissues (gm./100 gm. of lipide-free, dry tissue)*

Treatment	No. of experiments	Liver		Tumor	
		RNA	DNA	RNA	DNA
Control	18	5.35 ± 0.72	2.35 ± 0.31	6.62 ± 1.53	5.88 ± 1.02
AH(150) alone	4	5.43 ± 0.97	4.03 ± 2.07	6.00 ± 2.29	6.66 ± 1.55
DP(150) alone	4	6.03 ± 1.25	3.90 ± 2.05	4.66 ± 2.40	5.05 ± 1.26
AH(150)+ DP(150)	13	5.57 ± 0.66	1.95 ± 0.61	6.94 ± 1.23	4.76 ± 0.88

TABLE 8.—*RNA and DNA content of liver and tumor tissue (values in percent greater or less than the control value)*

Treatment and dosage	No. of experiments	Liver		Tumor	
		RNA	DNA	RNA	DNA
AH-150	3	−6.8	−15.2	−8.15	−15.1
DP-150	4	+17.33	−5.06	−2.11	−19.6
AH-150 + DP-150	13	+6.90	−10.9	−2.15	−20.8

ments. Variation among analyses in each of the single treatment groups of only 4 experiments each were too great to yield a reliable average value.

The data of table 8 were obtained by comparing the results of the treated animals with their respective controls in each experiment. The differences from the control values, expressed as percent, were then averaged to obtain the values cited. Whether one calculates these differences as described or from the data given in table 7, approximately the same values result for those animals which received both drugs. For the single drug treatments large discrepancies result in liver DNA and tumor RNA values.

There was a drop in the DNA content of both the liver and tumor tissue when either agent was used alone or in combination. The effect was most pronounced when combination therapy was used. The values are expressed as percentage increase or decrease in RNA and DNA of liver and tumor tissue compared to the control. The average value expressed as percentage greater or less than the control value obtained for each constituent from all experiments is recorded. The experimental conditions for therapy were the same as those described under "Methods." Therapy consisted of 9 successive daily injections of the drug or drugs.

In all cases these experiments indicated a decrease in DNA in the treated animals which was greater in the tumor tissue than in the liver tissue. RNA values were lower than the controls in the tumor tissues but to a lesser extent. In liver tissue they were lower in a limited number of experiments where aspartyl hydrazine was used alone. With deoxypyridoxine or with the combination treatment where there was a greater number of experiments the values showed an increase over the controls.

These data would indicate that the drug therapy does indeed interfere with nucleic acid synthesis and particularly DNA synthesis. The effect was more pronounced on the tumor than liver tissue. This may indicate some degree of selectivity for the tumor tissue.

The increase in RNA content of the liver tissue is of interest. The range of variation among these 13 experiments was from +30.8 to −17.1 percent from the controls, with 7 of the 13 experiments showing higher and 6 showing lower values for RNA than the controls. Eight out of 13 of the RNA values on tumor tissue were lower than the controls, 2 showed no difference and 3 were higher than the controls with a range from −18.7 to +21.4. DNA on the liver samples ranged from −43 to +56 with 9 less and 4 greater than the control. DNA in the tumor tissue showed a range from −41 to +8, with 12 less than the controls and one experiment where the value was greater. Though an attempt was made to obtain a representative piece of tissue, approximately 0.1 gm. from each animal's tumor or liver, the variation in composition within a tissue sample may have been responsible for the large variations in the content of the nucleic acid components between experiments.

In seeking an explanation for the higher than control values for RNA in the treated animals, the work of Cohen (*22*, *23*) may be pertinent. In the case of T_{15} thymineless mutant of *Escherichia coli*, the absence of thymine in the medium, while preventing DNA synthesis, did not prevent the continued synthesis of RNA.

Discussion

It is evident that aspartyl hydrazine does have a mild inhibitory effect on the growth of sarcoma 180 under the conditions of these experiments. It is not, however, very selective for the tumor tissue. The effect obtained with it is enhanced considerably by the aspartic-glutamic transaminase inhibitor, deoxypyridoxine. This combination of drugs can reduce the tumor growth as much as 80 percent over the untreated controls for a rather limited period of treatment. The tumor tissue then resumes a rapid growth and, whether or not drug treatment is continued, the animals develop large tumors and die without much extension of survival time.

Aspartyl hydrazine also exerts a strong and noncompetitive inhibition of the utilization of aspartic acid by *Lactobacillus delbrueckii*. Data with this organism show, however, that continued cultivation in the presence of aspartyl hydrazine results in the emergence of a culture which has a resistance to the inhibitor (table 5), presumably the same effect as was noted with the tumor.

The fact that aspartic acid is an obligatory intermediate for nucleic acid synthesis may account for the lack of selectivity. In other words, the requirements of the nucleic acid building stones are too nearly the same for the normal as they are for the tumor tissues. It may be that the only selectivity is that due to the difference in the rate of growth of the two types of tissues. The data on analysis of tumor tissues and non-tumor tissues of tumor-bearing mice suggested a quantitative difference in re-

quirements for aspartic acid that could furnish a rational basis for therapy with aspartic acid analogues. Other analogues of aspartic acid may fulfill the requirement as a blocking agent more satisfactorily. However, α-methylaspartic acid and hydroxyaspartic acid, though inhibiting aspartic acid utilization by *L. delbrueckii* have their effect rather easily reversed by aspartic acid. α-Methylaspartic acid, under the conditions in which it was tested, did not inhibit the growth of sarcoma 180.

L-Glutamyl hydrazine and DL-histidine hydrazine alone, in contrast to aspartyl hydrazine, showed practically no tumor inhibition, however, when combined with deoxypyridoxine, the tumor inhibition was equal to and perhaps superior to that obtained with aspartyl hydrazine. The function of L-glutamine as a precursor for two of the nitrogens of the purine ring may explain the antagonistic action of L-glutamyl hydrazine. The fact that it is without effect in the absence of deoxypyridoxine supports the belief that L-glutamine may arise *via* glutamic-aspartic transaminase.

When the hydrazines of both aspartic and glutamic acid are used simultaneously with deoxypyridoxine it would seem that more effective tumor growth inhibition should be obtained because one would presumably be attacking three reactions in the pathway of purine synthesis at one time. It was necessary to reduce the dosage level of each drug somewhat in order to prevent animal loss, and the net result on tumor inhibition was about the same as the maximum effect obtained with either hydrazine alone when coupled with deoxypyridoxine.

A somewhat different explanation for the action of deoxypyridoxine and acid hydrazides on sarcoma 180 was given by Brockman *et al.* (*24*). They described the effects of a number of acid hydrazides, in combination with 4-deoxypyridoxine, and alone on sarcoma 180 in mice on complete and vitamin B_6-deficient diets. They reported deoxypyridoxine to be without effect on the growth of the tumor in animals on a complete diet but that it was effective as a tumor inhibitor in animals on a B_6-deficient diet.

They found that a number of acid hydrazides were capable of inhibiting growth of the tumor in B_6-deficient mice and that vitamins of the B_6 group prevent this inhibition. They attributed the effect to a nonspecific reaction of the acid hydrazide with pyridoxal to form an inactive conjugate which resulted in further depletion of the B_6 content of the tissues with resulting tumor inhibition.

Azaserine or amethopterin, both of which interfere with the biosynthesis of purines in the pigeon liver system, enhance the effect of either aspartyl or glutamyl hydrazine and deoxypyridoxine. With azaserine the maximum inhibitory effect produced on tumor growth was about 95 percent. Amethopterin because of toxicity with this combination, was used at a reduced level, and the enhancement of inhibition by this compound was not great. 6-Mercaptopurine also supplemented this combination with reasonable effectiveness (*see* table 6).

An interesting effect of the various drug combinations was on nucleic acid synthesis. There was less DNA per unit of lipide-free dry tissue in both the liver and tumor tissues of the treated animals in all cases. It

appears that the aspartyl hydrazine and deoxypyridoxine does depress DNA synthesis. In general, the DNA content of the tumor or liver tissue was least where tumor tissue growth was the least. The addition of a third drug, such as azaserine, amethopterin, or 6-mercaptopurine, to the combination accentuated the differences and also increased the tumor inhibition.

Summary

Under the experimental conditions used, DL-β-aspartyl hydrazine inhibited the growth of sarcoma 180 in mice to about 40 percent that of the control animals. When combined with 4-deoxypyridoxine the effect on tumor inhibition was about 80 percent. Both drugs were used at approximately the maximum tolerated dosage to obtain this effect. When the treatment was withdrawn after 10 days, tumors began to grow again. With a prolonged treatment, the tumor tissue was no longer responsive to the drugs after a period of 2 weeks. Survival time of treated animals was not extended greatly beyond that of the controls.

Lactobacillus delbrueckii NRRL-LD5, whose aspartic acid utilization was irreversibly inhibited by DL-aspartyl hydrazine, developed a resistance to the drug upon continued transfer in its presence and eventually the organism was able to use the aspartyl hydrazine to satisfy its aspartic acid requirements. Preliminary evidence indicates that *L. delbrueckii* removes ammonia from the hydrazine portion of the molecule.

L-Glutamyl hydrazine by itself had no appreciable effect on tumor growth but when combined with deoxypyridoxine it gave results comparable to those with aspartyl hydrazine. DL-Histidine hydrazine monoacetate behaved similarly.

Various combinations of the 3 aforementioned drugs and these combinations supplemented with other compounds, such as azaserine, amethopterin, and 6-mercaptopurine, were also tested.

Nucleic acid analyses on the liver and tumor tissues indicated a decreased amount of DNA in the tumor tissue and to a lesser extent in the liver tissue of the animals subjected to therapy. RNA values were slightly lower than the controls in the tumor tissue and also in the liver tissue when aspartyl hydrazine was used alone. RNA values in the liver tissue of animals treated with deoxypyridoxine or the combination treatment were, however, higher than those of the controls.

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Immunogenetic Properties of Tumors That Have Acquired Homotransplantability [1]

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Studies on immunological tolerance have shown that it is possible to induce in animals a specific unresponsiveness to tissue grafts with a foreign genetic constitution, by treating fetuses (or newborn) with the antigenic material responsible for transplantation immunity (*1*). In experiments on the induction of tolerance in ICR Swiss mice to the 6C3HED and DBA ascites tumors (by inoculating tumor cells into ICR fetuses or ICR adults treated *in utero* with blood), Koprowski (*2*, *3*) found that not only was there a change in the response of the treated hosts but that these strain-specific tumors had been adapted to give successful homografts. They could now grow progressively in untreated ICR mice and in other foreign strains. In contrast to the results obtained with nonmalignant tissues, these tumors had changed as a result of growing in the treated hosts. It is therefore of considerable interest to try and determine the mechanism responsible for these changes. Since homotransplantability is found among tumors and is not a property of comparable normal tissues, it is also of interest to determine the degree to which the mechanism of homotransplantability in the adapted tumors is similar to the suggested loss of antigenicity (*4–6*), or to the gain of resistance to the immune response found in some other homotransplantable tumors that had previously been analyzed (*7*, *8*).

The present immunogenetic study of original and adapted sublines of the 6C3HED and DBA ascites tumors was therefore undertaken in order to try and elucidate both the mechanism responsible for homotransplantability of the adapted tumors and the nature of the adaptation.

Material

The ascites tumors used in the present investigation were the MC1A sarcoma, 6C3HED lymphosarcoma, DBA lymphoma, and the sublines of 6C3HED and DBA that had been adapted to ICR Swiss mice. The MC1A and 6C3HED tumors, also used in previous studies (*7*, *8*) had been obtained from Dr. G. Klein, and we are very much indebted to Dr. H. Koprowski for supplying us with the original DBA and the adapted

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6C3HED-ICR and DBA-ICR. The adapted subline of 6C3HED-ICR used in the present study is the one obtained by Koprowski after inoculation directly into ICR fetuses and referred to by him as subline 123 (*3*).

Unless otherwise stated, 6C3HED, MC1A, and DBA were routinely maintained in hosts belonging to the strain of origin, and the adapted sublines either in ICR Swiss or in our own line of Swiss mice.

The C3H, DBA/2, C57BL, BALB/c, and Swiss mice are from the same sources as those used previously (*7–9*), and the ICR strain of Swiss was obtained through the courtesy of Dr. H. Koprowski. In confirmation of Koprowski's results (*3*) table 1 shows the clear difference in homotransplantability between the original 6C3HED and DBA, and the adapted 6C3HED-ICR and DBA-ICR when tested in doses of about 20×10^6 cells in our strains of mice.

TABLE 1.—*The homotransplantability of 6C3HED, 6C3HED-ICR, DBA, and DBA-ICR*

Tumor	Site*	Host strain tested (name and *H-2* pseudoallele)			
		C3H *H-2^K*	DBA/2 *H-2^D*	C57BL *H-2^B*	Swiss *H-2^S*
6C3HED	IP	+	−	−	− †
	IM	+	−	−	−
6C3HED-ICR	IP	+	+	+	+
	IM	+	+	+	+
DBA	IP	−	+	−	−
	IM	−	+	−	−
DBA-ICR	IP	+	+	+	+
	IM	+	+	+	+

*IP = intraperitoneal inoculation; IM = intramuscular inoculation.
† + = Lethal growth of tumor; − = regression of tumor.

Experimental

The immunological properties of the original and ICR-adapted tumors have been studied from the following points of view:

1) Their antigenicity as shown by the production of isohemagglutinins.
2) The antigenic components of the tumor-cell agglutinogens.
3) The cytotoxic immune response involved in the homograft reaction.

Production of Isohemagglutinins by Homografts of Original and ICR-Adapted Tumors

Hemagglutinins produced by tumor homografts were determined according to the serological method of Gorer and Mikulska (*10*). Dilutions

TABLE 2.—*Hemagglutinins produced by 1 intraperitoneal inoculation of 6C3HED and 6C3HED-ICR into C57BL mice**

Tumor	Antiserum dilution 1:										
	2	2^2	2^3	2^4	2^5	2^6	2^7	2^8	2^9	2^{10}	2^{11}
6C3HED.....	++	+++	+++	++	++	++	++	+|	+	±	−
6C3HED-ICR..	+++	+++	+++	++	++	++	++	+|	+	±	−
Control: normal C57BL serum.	−	−	−	−	−	−	−	−	−	−	−

*| = Half value of +.

of antisera were made in 3 percent Dextran (Intradex salt-free, Glaxo) and the erythrocytes were suspended in inactivated human serum, previously absorbed with mice red blood cells, and diluted 1 : 0.5 in saline. The red-cell suspension and antisera were incubated at 37° C. for 1 hour.

In the first experiment a determination was made of the hemagglutinins produced by one tumor inoculation.

Two groups, each of 6 C57BL mice, were inoculated intraperitoneally with 15×10^6 cells of 6C3HED and 6C3HED-ICR, respectively. On the 7th day after inoculation the ascites fluid was washed out (*8*), in order to avoid the absorption of antibodies by the growing tumor and differences in antibody production due to growth differences of the various sublines. Seven days later, *i.e.*, on the 14th day following inoculation, the animals were bled and the pooled antisera was tested for agglutinins against C3H erythrocytes. The results, given in table 2, show that both tumors elicited in C57BL mice the same level of isohemagglutinins.

Similar tests were carried out with antisera produced by intramuscular inoculations of the 2 tumors into C57BL, DBA/2, and Swiss mice. In this site of growth the tumors could not be properly removed before the bleeding. The animals inoculated with 6C3HED-ICR, therefore, had growing solid tumors when they were bled, whereas in those inoculated with the strain-specific 6C3HED the tumor had already regressed. The results, given in table 3, show a somewhat higher agglutinin level for the

TABLE 3.—*Hemagglutinins produced by 1 intramuscular inoculation of 6C3HED and 6C3HED-ICR into C57BL, DBA/2, and Swiss mice*

Tumor	Host strain	Titer 1:
6C3HED	C57BL	4096
6C3HED-ICR		1024–2048
6C3HED	DBA/2	4096
6C3HED-ICR		1024–2048
6C3HED	Swiss	4096
6C3HED ICR		<128

original 6C3HED than for the adapted tumor. Since in this case an *in vivo* absorption by the growing adapted tumor may be expected, this may explain the decreased level of free agglutinins with this tumor at this inoculation site. The low level of free agglutinins produced by 6C3HED-ICR in Swiss may be due to a higher absorption by the growing tumor owing to a higher growth rate in these mice.

A comparison of agglutinins produced by 1 and by 2 intraperitoneal inoculations of DBA and DBA-ICR was also made with C57BL mice as antibody producers. The 1-inoculation experiment was carried out in the same manner as that described for 6C3HED and 6C3HED-ICR, and the results are given in table 4.

In the 2-inoculation experiment, the ascites fluid of the first inoculation was washed out after 7 days and the animals were reinjected on the same day with 15×10^6 tumor cells. In the animals inoculated with the ICR subline, the tumor had regrown after the 2nd inoculation; the ascites fluid was therefore again washed out 3 days prior to bleeding, in order to avoid *in vivo* absorption. The results of these experiments, which are also given in table 4, show that both the original and ICR subline again elicited the same amount of agglutinins.

In order to determine whether adaptation of tumors in animals of the ICR strain involved the assimilation of antigenic components from the ICR hosts, 6C3HED-ICR was tested for ICR agglutinogens. Two lots of antisera produced in C57BL by inoculations of 6C3HED-ICR, one with a titer of 2^{11} and the other with a titer of 2^6 against C3H red blood cells, were tested for agglutinins against erythrocytes of ICR mice. There was no evidence of an incorporation of ICR antigens into the adapted tumor cells.

Antigenic Constitution of Original and Adapted Tumors

The above data have shown that there were no differences between the original and the adapted tumors in their capacity to elicit agglutinins against erythrocytes of their strain of origin. Further experiments were carried out to determine whether the two sublines of each tumor differ in their antigenic components. This problem can be investigated by the differential absorption of red blood cell agglutinins (*5*, *6*, *11*, *12*), and this was therefore the method that was used in the present experiments.

In the first experiment, antiserum was prepared in DBA/2 mice against MC1M, an ascites tumor that also originated in C3H and that had previously been shown to be a good anti-C3H antibody producer (*8*). This antiserum was then absorbed: 1) with cells of the original 6C3HED, 2) with 6C3HED-ICR grown in C3H, and 3) with 6C3HED-ICR grown in DBA/2 mice for one transplant generation. One sample of the antiserum was left unabsorbed as a control. The purpose of absorption with 6C3HED-ICR grown in a foreign strain was to determine whether these cells will show a lower absorption in comparison to tumor cells taken from the strain of origin, since such a difference would show that the cells of the ICR subline had been sensitized with agglutinins produced by the

TABLE 4.—*Hemagglutinins produced by 1 and 2 intraperitoneal inoculations of DBA and DBA-ICR into C57BL mice**

	Antiserum dilution 1:													
	2	2^2	2^3	2^4	2^5	2^6	2^7	2^8	2^9	2^{10}	2^{11}	2^{12}	2^{13}	2^{14}
DBA: 1 inoculation.....	++	+++	+++	+++	+++	+++	++	++	+|	+|	+|	+	+	−
DBA-ICR: 1 inoculation...	+++	+++	+++	+++	++	++	++	+	+	++	++	+	+	−
DBA: 2 inoculations.....	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	±	−
DBA-ICR: 2 inoculations..	+++	+++	+++	+++	+++	+++	+++	++|	++|	++	+	+	±	−
Control: normal C57BL serum.....	−	−	−	−	−	−	−	−	−	−	−	−	−	−

*| = Half value of +.

TABLE 5.—*Results of 1* in vitro *absorption of anti-MC1M hemagglutinins produced in DBA/2 mice**

Absorbed with cells of:	Antiserum dilution 1:																		
	2	2^2	2^3	2^4	2^5	2^6	2^7	2^8	2^9	2^{10}	2^{11}	2^{12}	2^{13}	2^{14}	2^{15}	2^{16}	2^{17}	2^{18}	2^{19}
(Unabsorbed)	−	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+|+	++	++	+	−
6C3HED-ICR from DBA	−	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	−	−	−
6C3HED-ICR from C3H	+	+++	+++	+++	+++	+++	++	++	++	++	++	++	+	+	−	−	−	−	−
6C3HED from C3H	+	+++	+++	+++	+++	+++	++	++	+	−	−	−	−	−	−	−	−	−	−
Control: normal DBA/2 serum	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

*| = Half value of +.

foreign host. Tumor cells for the absorption tests were washed in 4 percent citrate and then several times in saline. In this first experiment the antisera were absorbed once with equal volumes of tumor cells at 37° C. for 1 hour. It has been found that with one such absorption, there is only a partial removal of antibodies. However, even such an incomplete absorption (table 5), shows that the greatest absorption was obtained with the original 6C3HED cells which have decreased the agglutinin level from 2^{19} of the control serum to 2^{9}. The 6C3HED-ICR cells grown in C3H have absorbed to a lesser extent leaving a titer of 2^{14}, while cells of the same ICR subline taken from DBA/2 hosts still gave a titer of 2^{17} and thus showed only a slight absorption of the agglutinins. These results show: 1) that cells of the original, strain-specific, 6C3HED tumor have a much greater capacity to absorb anti-MC1M agglutinins than cells of the ICR subline, and 2) that tumor cells of the ICR subline, while growing progressively in hosts of foreign strains are sensitized with agglutinins. That such an *in vivo* sensitization can take place has also been confirmed by the difference in absorptive capacity of the homotransplantable tumors MC1M and MC1A, depending on whether the cells had been growing in C3H mice, the strain of origin, or in foreign C57BL hosts for one transplant generation (table 6). The results in table 6 were obtained after a single absorption of anti-MC1M serum produced in C57BL mice.

Further tests were then performed with the aim of obtaining a complete absorption. The tumors tested were the original 6C3HED, 6C3HED-ICR, the original DBA, and DBA-ICR. The antiserum for absorption by the first 2 tumors was produced by inoculations of MC1M cells into C57BL mice. The antiserum for the second 2 tumors was produced by inoculation of DBA into C3H mice. In these tests each sample of antiserum was absorbed twice with ascites cells, each time for 1 hour at 37° C., and this method of absorption has also been used in all the following tests. The cells for absorption were taken from tumors grown in their strain of origin, *i.e.*, cells of the original 6C3HED and the ICR subline

TABLE 6.—In vivo *sensitization of tumor cells by hemagglutinins during growth for 1 transplant generation in a foreign host*

Absorbed with cells of:	Titer 1:
(Unabsorbed)	256
MC1A from C3H	8
MC1A from C57BL	128
MC1M from C3H	8
MC1M from C57BL	128
6C3HED from C3H	64
6C3HED from C57BL	128

TABLE 7.—*Results of 2 in vitro absorptions of*

Antiserum	Absorbed with cells of	Antiserum dilution 1:					
		2	2^2	2^3	2^4	2^5	2^6
C57BL Anti-MC1M	(Unabsorbed)	++	++	++	+++	+++	+++
	6C3HED	±	+	±	−	−	−
	6C3HED-ICR from C3H	+++	+++	+++	+++	+++	+++
C3H Anti-DBA	(Unabsorbed)		++	++	++	+++	+++
	DBA	−	−	−	−	−	−
	DBA-ICR from DBA	+++	+++	+++	+++	+++	+++

were taken from C3H mice, and cells of DBA and DBA-ICR from DBA hosts. This was done in order to avoid any possible effects of antibody coating of cells taken from ICR hosts. The absorbed sera were tested for agglutinins against C3H and DBA erythrocytes, respectively. The results, given in table 7, clearly show that the original tumors completely absorbed the agglutinins from the antisera against which they were tested, whereas cells of the adapted sublines of both tumors gave a very much lower absorption. The cells of the ICR tumors only decreased the titer of the anti-C3H serum from 2^{18} to 2^{14} and that of the anti-DBA serum from 2^{17} to 2^{11}.

This lack of absorption by the ICR tumor cells can be attributed either to a low absorptive capacity of the adapted tumors as a result of masking of antigens that exist but are located in a nonreactive position on the cell membrane, or to a complete loss in the adapted tumors of one or more antigens possessed by the original tumor. Further absorption experiments were therefore carried out to test these two possibilities. The assumption was that if the low absorption shown by the adapted tumors was due to a low absorptive capacity of the cells and not due to a lack of antigen(s), then it would be expected that these cells would not even absorb hemagglutinins produced by the ICR-adapted tumors themselves. On the other hand, if cells of the ICR sublines did not absorb all the antibodies because they lack one or more antigens possessed by the original tumor and against which antibodies exist in the serum, then they should completely absorb their own antiserum, although they would neither produce nor absorb all the isoantibodies produced by cells of the original tumor.

Two series of parallel experiments were therefore carried out for the 6C3HED and the DBA original and adapted tumors. Antisera were prepared in C57BL mice against all 4 tumors. The anti-6C3HED was divided into 4 samples. One was absorbed twice with the original 6C3HED tumor. The second was similarly absorbed with cells of 6C3HED-ICR that had been growing in C3H mice for 8 transplant generations, *i.e.*, for 56 days, and the third with 6C3HED-ICR cells that had always been grown in our Swiss or the ICR Swiss mice. In all

*hemagglutinins by cells of original and ICR sublines**

Antiserum dilution 1—Continued											
2^7	2^8	2^9	2^{10}	2^{11}	2^{12}	2^{13}	2^{14}	2^{15}	2^{16}	2^{17}	2^{18}
+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
−	−	−	−	−	−	−	−	−	−	−	−
+++	+++	+++	++	++	++	++	+				
+++	+++	+++	+++	+++	++|	++	++	+	+	±	−
−	−	−	−	−	−	−	−	−	−	−	−
++|	++	+	+	±	−						

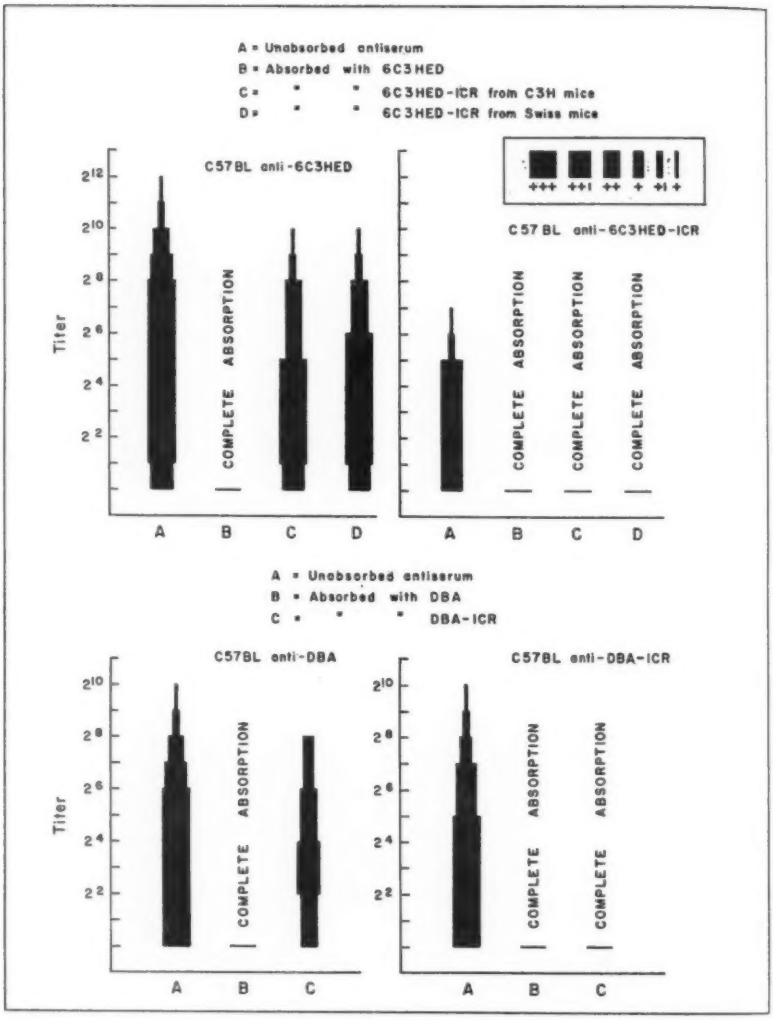
*\| = Half value of +

these experiments, the ascites cells used for absorption were taken from 7-day-old tumors. The fourth sample was left unabsorbed as a control. A similar set of absorption was carried out with anti-6C3HED-ICR. The antisera were then tested for agglutinins against C3H erythrocytes. The results (text-fig. 1) show that 6C3HED cells completely absorbed anti-6C3HED serum and anti-6C3HED-ICR serum, whereas the 6C3HED-ICR cells completely absorbed their own antiserum but showed only a slight absorption of the anti-6C3HED. Similar results were obtained with 6C3HED-ICR cells grown in C3H or in Swiss mice, so that there was no sensitization of the cells in the Swiss mice in this experiment.

These results, therefore, show that the ICR-adapted 6C3HED is not devoid of the capacity to carry out *in vitro* absorption of hemagglutinins, as it has completely absorbed isoantibodies produced by the adapted tumor. The original 6C3HED has also completely absorbed anti-6C3HED-ICR, and this shows that the original 6C3HED contains all agglutinogens possessed by the adapted tumor. On the other hand, the ICR subline has not absorbed all the anti-6C3HED agglutinins, although these were removed by the original 6C3HED cells. It follows, therefore, that the anti-6C3HED serum contains antibodies against at least one antigen which the adapted tumor does not contain. The adaptation in the ICR strain was thus accompanied by a loss of at least one agglutinogen of the original tumor.

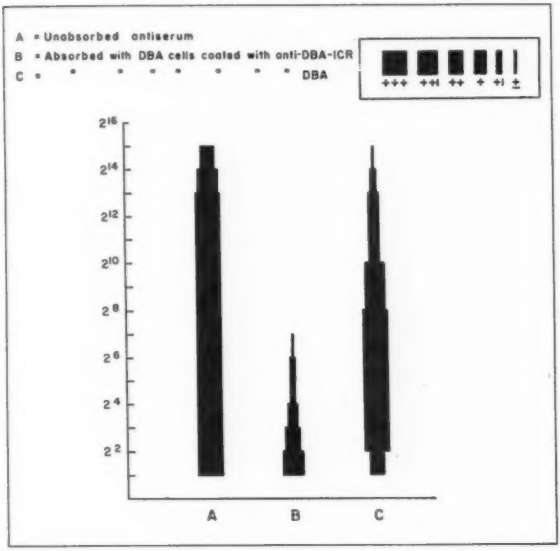
Similar results have been found for the DBA original and adapted tumors. In this case also (text-fig. 1) the DBA cells absorbed all agglutinins of the anti-DBA and anti-DBA-ICR, whereas the adapted DBA absorbed completely the anti-DBA-ICR serum, but showed only a slight absorption of anti-DBA. The ICR line must also, therefore, have lost at least one antigen of the original tumor.

A further demonstration of this antigenic difference between the original and adapted tumors was based on the assumption that if the original tumor contains more isoantigens than the adapted one, then the cells of, *e.g.*, the original DBA after absorption of anti-DBA serum should not absorb any more agglutinins when suspended again in another sample of



TEXT-FIGURE 1.—The *in vitro* absorption of antisera by cells of the original and adapted tumors.

anti-DBA. On the other hand, DBA cells, after absorption of anti-DBA-ICR serum, if put into anti-DBA, should carry on absorbing those agglutinins which do not exist in the anti-DBA-ICR. This expectation was confirmed by an experiment that showed (text-fig. 2) that coating of the DBA cells with anti-DBA-ICR does not saturate all the antigenic sites of their cell membrane, whereas coating these cells with anti-DBA causes a complete saturation of the antigens. The anti-DBA, therefore, contains antibodies toward antigens which do not react with anti-DBA-ICR.



TEXT-FIGURE 2.—Results of coating DBA cells by anti-DBA and anti-DBA-ICR hemagglutinins.

In the absorption experiments given in text-figure 1, it has been shown that when cells of 6C3HED-ICR grown in Swiss mice for 7 days were checked for their capacity to absorb *in vitro* anti-6C3HED-ICR agglutinins, no sensitization of these cells could be shown. The cells from C3H and from Swiss, have both completely absorbed the anti-6C3HED-ICR antiserum. However, since these cells were grown in the foreign hosts for only 7 days, this may be too short a time for demonstrating sensitization, even if it does take place in Swiss hosts.

A further experiment was therefore carried out in which anti-6C3HED-ICR was absorbed by 6C3HED-ICR cells growing in C3H, BALB/c, and Swiss mice for 11 days. The results, given in table 8, show that whereas cells of the 6C3HED-ICR tumor taken from C3H hosts have carried out a complete absorption of anti-6C3HED-ICR agglutinins, comparable

TABLE 8.—*Results of 2* in vitro *absorptions of C57BL anti-6C3HED-ICR hemagglutinins by cells of 6C3HED-ICR grown in the original and foreign strains for 11 days*

Absorbed with cells from:	Antiserum dilution 1:									
	2^2	2^3	2^4	2^5	2^6	2^7	2^8	2^9	2^{10}	2^{11}
(Unabsorbed)	+++	+++	+++	+++	+++	+++	++	+	+	−
C3H hosts	−	−	−	−	−	−	−	−	−	−
Swiss hosts	+++	++	++	+	±	−	−	−	−	−
BALB/c hosts	+++	++	++	+	+	+	−	−	−	−

amounts of cells from BALB/c and Swiss hosts did not give a complete absorption. It has thus been shown that 6C3HED-ICR cells grown in Swiss mice for 11 days were already coated with agglutinins produced in these hosts.

Identification of Antigens

Since the *in vitro* absorption experiments demonstrated that the adapted tumors lost at least one agglutinogen of the original antigenic complex, an attempt was made to determine which of these antigens had been lost. It has been shown with regard to the *H-2* system of the mouse that C3H have the antigenic constitution ACdEfHK (*11–13*), *i.e.*, these mice contain at least the agglutinogens ACEHK. We are very much indebted to Dr. D. B. Amos for supplying antisera that included either anti-CD, anti-BE, or anti-DK so that it was possible to test for the individual antigens C, E, and K. Each antiserum was divided into 3 samples. One was absorbed with 6C3HED cells, the second with 6C3HED-ICR cells, and the third was left unabsorbed as a control. The absorbed and control antisera were then tested for agglutinins against C3H erythrocytes. The results, given in table 9, show that both the original and adapted tumors completely removed the anti-C and anti-K agglutinins, whereas anti-E was absorbed by the original 6C3HED, but not by 6C3HED-ICR. It can be concluded therefore that the adapted 6C3HED-ICR still has the C and K antigens, but that it has lost the E agglutinogen. It has been shown in the previous section (text-fig. 1) that the adapted tumor did not absorb anti-6C3HED serum produced in C57BL mice. This cannot, however, be attributed to the existence of E antibodies in that antiserum, since C57BL mice contain the E antigen (*11–13*) and they are therefore incapable of producing anti-E. It can be deduced that in addition to E, at least one more antigen is missing in the adapted tumor.

An experiment was also carried out with the original and adapted DBA. The *H-2* system of the DBA/2 strain of mice has the antigenic constitution aCDEdFkH (*11–13*). In this case, however, the only antiserum available was anti-CD. Absorption of this antiserum with cells of the original and adapted DBA (table 10) showed that both can absorb the C and D agglutinins, so that it is neither C nor D that is missing in the adapted tumor. It can be presumed that it is at least one of the other agglutinogens of DBA/2 that is absent in the DBA-ICR cells.

Homograft Reaction to Transplants of Adapted Tumors

The homograft reaction, *i.e.*, the cytotoxic immune response elicited by the transplant, was studied by evaluating the increase in host resistance to a second tumor graft evoked by a first transplant. Homografts of the adapted tumors gave lethal growth at all inoculation sites (intraperitoneal, intramuscular, and subcutaneous) in all strains of mice tested. The immunizing properties of the successful homografts were therefore studied by washing out the first graft 7 days after intraperitoneal inoculations, and then reinoculating intraperitoneally at various times after the washing,

TABLE 9.—In vitro *absorption tests for the agglutinogens C, E, and K in 6C3HED and 6C3HED-ICR**

Antiserum	Absorbed with cells of:	Antiserum dilution 1:									
		2^3	2^4	2^5	2^6	2^7	2^8	2^9	2^{10}	2^{11}	2^{12}
Anti-CD	(Unabsorbed)	+++	+++	++|	++|	++	+	±	−	−	−
	6C3HED	−	−	−	−	−	−	−	−	−	−
	6C3HED-ICR	−	−	−	−	−	−	−	−	−	−
Anti-BE	(Unabsorbed)	+++	+++	+++	+++	+++	++	+	±	−	−
	6C3HED	−	−	−	−	−	−	−	−	−	−
	6C3HED-ICR	++	+++	+++	+++	++|	++	±	−	−	−
Anti-DK	(Unabsorbed)	+++	+++	+++	+++	+++	++|	++|	+|	+	−
	6C3HED	−	−	−	−	−	−	−	−	−	−
	6C3HED-ICR	−	−	−	−	−	−	−	−	−	−

*| = Half value of +.

TABLE 10.—In vitro *absorption tests for agglutinogens C and D in DBA and DBA-ICR*

Antiserum	Absorbed with cells of:	Antiserum dilution 1:										
		2^4	2^5	2^6	2^7	2^8	2^9	2^{10}	2^{11}	2^{12}	2^{13}	2^{14}
Anti-CD	(Unabsorbed)	+++	+++	+++	+++	+++	+++	++	+	+	±	−
	DBA	−	−	−	−	−	−	−	−	−	−	−
	DBA-ICR	−	−	−	−	−	−	−	−	−	−	−

either with the same or another homotransplantable tumor. Regression of the second transplant was then attributed to the immunizing effect of the first graft. This experimental approach could not, however, be used with Swiss mice, since washing out of the adapted tumors on the 7th day was followed by an almost immediate regrowth, so that the peritoneum was already reswollen 3 to 4 days after the washing. It is of interest to note that Swiss is the strain of mice in which these tumors were adapted and in which they have been growing for many transplant generations.

The objects of the transplantation experiments were: 1) to test whether successful homografts of the ICR tumors elicit transplantation immunity although they are not rejected by the host; 2) to compare the strength of this immune response to that evoked by the original tumors; 3) to compare the transplantation immunity caused by 6C3HED-ICR to that of the MC1A ascites tumor, another homotransplantable tumor originating in C3H whose properties had previously been studied (*7*, *8*); 4) to deduce from these tests the factors determining the homotransplantability of the adapted tumors, *i.e.*, whether the increased transplantability is a result of a decreased incompatibility due to a lower antigenicity of the tumor or to an increased resistance of the adapted tumor to the homograft reaction.

The results of the transplantation tests with 6C3HED, 6C3HED-ICR, and MC1A are given in table 11. In all the experiments inoculation doses were about 15 to 20 $\times$ 10^6 cells.

The data show that 6C3HED-ICR elicits both a cytotoxic host response when growing in a foreign host and that it is itself to some extent sus-

TABLE 11.—*Effects of a first graft of 6C3HED, 6C3HED-ICR and MC1A, on progressive growth of a second graft of 6C3HED-ICR and MC1A in foreign hosts*

First graft	Second graft	Days between 1st and 2nd graft	Host strain	Progressive growth of 2nd graft
6C3HED	MC1A	7	C57BL	4/5*
		10	C57BL	5/5
		14	DBA/2	5/5
6C3HED-ICR	MC1A	7	C57BL	0/6
		10	C57BL	2/6
		10	DBA/2	0/6
		14	DBA/2	0/5
MC1A	6C3HED-ICR	10	C57BL	1/6
MC1A	MC1A	10	C57BL	0/6
6C3HED-ICR	6C3HED-ICR	7	C57BL	3/4
		10	C57BL	6/6
		10	DBA/2	6/6
		14	DBA/2	4/4
		19	C57BL	5/5
6C3HED	6C3HED-ICR	7	C57BL	5/5
		10	C57BL	6/6
		14	DBA/2	4/4
		19	C57BL	5/5

*The denominator shows the number of animals grafted, and the numerator the number of animals killed by progressive growth of the second graft.

ceptible to this immunity. That 6C3HED-ICR elicits an immune response is shown by the data that a first graft of 6C3HED-ICR could prevent the growth of a subsequent graft of MC1A. In nearly all the 6C3HED-ICR followed by MC1A experiments, 6C3HED-ICR regrew after the washing. The failure of MC1A to grow progressively in these animals could therefore be attributed either to an immune response produced by the first graft, or to the result of competition between MC1A and the 6C3HED-ICR, which had regrown from the cells left after the washing. To test for such a possible competitive effect, DBA/2 mice were inoculated simultaneously with cells of 6C3HED-ICR and MC1A and the developing ascites examined for the types of tumor cells present in the fluid. The cells of these 2 tumors can be readily distinguished by their chromosomes, since 6C3HED-ICR has a modal chromosome number of 45 and some very characteristically shaped chromosomes, whereas MC1A has a modal number of about 80 chromosomes (7). The results of this test showed that cells from both tumors were present after the mixed inoculations, so that the inability of MC1A to grow after a first graft of 6C3HED-ICR can be attributed to the immunizing effect of the first graft. That it is a question of immunity rather than competitive growth can also be supported by the fact that in 2 out of 6 C57BL, MC1A was able to grow progressively after a primary graft of 6C3HED-ICR. The immunity produced by 6C3HED-ICR was thus strong enough to prevent the growth of MC1A but it could not inhibit the regeneration of 6C3HED-ICR itself.

It is clear from the reverse experiment that 6C3HED-ICR is susceptible to the immunity produced by MC1A, and here even the progressive growth of 6C3HED-ICR in the one mouse with a tumor was due to the late development of a solid subcutaneous growth. The experiment with a 10-day interval between a first and second graft in C57BL also shows that 6C3HED-ICR can be susceptible to its own immunity. Three of 6 mice gave an intraperitoneal growth of the tumor, *i.e.*, growth at the site of inoculation, with a resulting death of the hosts at 12, 14, and 15 days after the second graft. But in the other 3 animals there was only a late development of a solid subcutaneous tumor, with a resulting death at 34, 35, and 47 days after the second graft. It can be presumed that this lack of an intraperitoneal growth and the extended survival time was caused by the immunity produced by the first graft. Similar results were obtained in the experiment in C57BL with a 10-day interval between a first graft of 6C3HED and a second graft of 6C3HED-ICR.

These transplantation experiments therefore show that 6C3HED-ICR can elicit an immune response since it can partially prevent its own growth and can almost completely prevent MC1A. But it produces a weaker immunity than MC1A, because MC1A can completely or almost completely prevent its own growth and that of 6C3HED-ICR.

The comparison of a first graft of 6C3HED followed by MC1A (where there is almost complete growth of MC1A) with a first graft of 6C3HED-ICR followed by MC1A (where there is almost complete inhibition of

MC1A) shows that the adapted 6C3HED-ICR gives a stronger homograft reaction than the original 6C3HED. It can thus be concluded that a homograft of 6C3HED-ICR not only produces a cytotoxic host response but that the response is even stronger than that produced by the original 6C3HED.

It is clear that the cytotoxic immune response produced by the original 6C3HED is strong enough to prevent its growth in foreign strains. On the other hand, the even stronger cytotoxic immunity produced by the adapted subline does not cause a regression of the tumor in foreign hosts. It can be deduced therefore that the capacity of the adapted tumor to resist its own homograft reaction must play an important role in its extreme homotransplantability.

The results of similar types of experiments with DBA and DBA-ICR are given in table 12, but in the absence of another homotransplantable tumor that originated in DBA/2, the experimental possibilities with this adapted tumor are much more limited than with 6C3HED-ICR. In the DBA followed by DBA-ICR grafts with a 10-day interval, and the DBA-ICR followed by DBA-ICR grafts with a 10- and 14-day interval, there was one mouse without any growth of DBA-ICR after the second graft and one mouse with an extended survival time. There is thus some evidence that DBA-ICR produces a cytotoxic immune response in a foreign host and that it is to some extent susceptible to the immune response produced by itself and DBA. The hemagglutinin titers have shown that DBA-ICR is not less antigenic than the strain-specific DBA in regard to the production of this type of antibody. Although agglutinins are not the antibodies responsible for the cytotoxic homograft reaction, this fact and the transplantation results make it likely that the ability to resist an immune response is also an important factor in allowing DBA-ICR to grow progressively in all foreign strains that have been tested.

The transfer of immunity by lymph nodes (*14*, *15*) suggests another approach to the problem of the nature of the homograft reaction produced by the adapted tumors. Koprowski *et al.* (*3*) had studied the effects of transplants of lymph nodes immunized with 6C3HED on a subsequent

TABLE 12.—*Effects of a first graft of DBA and DBA-ICR on progressive growth of a second graft of DBA-ICR in foreign hosts*

First graft	Second graft	Days between 1st and 2nd graft	Host strain	Progressive growth of 2nd graft
DBA	DBA-ICR	7	C57BL	5/5*
		10	C3H	5/5
		14	C3H	4/5
DBA-ICR	DBA-ICR	7	C57BL	4/4
		10	C3H	4/5
		14	C3H	4/5

*The denominator shows the number of animals grafted, and the numerator the number of animals killed by progressive growth of the second graft.

graft of 6C3HED-ICR. The present experiment was carried out to determine the behavior of lymph nodes from animals grafted with 6C3HED-ICR, since this would provide further evidence on the immunizing ability of the adapted tumor in a foreign host.

Fifteen DBA/2 mice were grafted subcutaneously on both sides with 6C3HED-ICR. Seven days later the axillary and inguinal lymph nodes were removed, cut into small pieces, and transplanted intraperitoneally into 5 DBA/2 at a dose of 18 nodes per animal. Two of these mice were then grafted on the same day, and the others 3 days later with 5×10^6 cells of 6C3HED-ICR; 4 untreated DBA/2 were similarly grafted as controls. The 4 control mice had survival times of 16, 19, 21, and 21 days, whereas in those previously grafted with lymph nodes 4 had survival times of 26, 27, 41, and 65 days, and one mouse is still surviving without a tumor 70 days after the graft of the tumor cells. This experiment therefore further shows that 6C3HED-ICR produces an immune response in a foreign host and that it is itself susceptible to the immune response which it produces.

Metastases

It has previously been pointed out that the procedure in our transplantation tests involved washing out of the tumor 7 days after inoculation. But it was found that this experimental procedure could not be applied in the case of 6C3HED transplanted into C3H mice. This tumor, when inoculated intraperitoneally, normally kills the C3H host in about 9 days. When the peritoneum was washed out on the 7th day, it was found that the animals died 2 days later, *i.e.*, at the same time at which they would have died had the tumor not been rinsed out. It seems, therefore, that the lethal effect of 6C3HED on C3H is not a result of a progressive growth of the intraperitoneal ascites fluid, but is rather due to metastases which have developed from the intraperitoneal ascites prior to the 7th day. Since no such phenomenon was found with 6C3HED-ICR (which kills the C3H host in about 18 days) a comparison was made of the metastasizing ability of the original and adapted subline.

Two groups of C3H mice were inoculated intraperitoneally with 6C3HED and 6C3HED-ICR, respectively. The animals of both groups were sacrificed on the 9th day, and pieces of liver, lung, and spleen were grafted into the muscle of other C3H mice, in order to check for metastases by the growth of tumors from these grafts. In both groups the tissues from each organ were grafted into 5 C3H mice. After 7 days the 6C3HED implanted tissues showed a fairly large tumor growth, whereas animals implanted with tissues of the 6C3HED-ICR carrying mice did not. The 6C3HED-ICR implanted tissues later developed tumors, showing that the adapted subline had also produced metastases, but the survival time of the 6C3HED-carrying animals was shorter than that of the 6C3HED-ICR mice. The results, given in table 13, show that at 25 days after inoculation 7 of 15 of the animals bearing 6C3HED metastases were dead, whereas all the 6C3HED-ICR animals were alive. Deaths totaled 73 percent with 6C3HED after 35 days, whereas this percentage

TABLE 13.—*Progressively growing tumors produced by intramuscular grafts of spleen, liver, and lung, taken from animals 9 days after an intraperitoneal inoculation of 6C3HED and 6C3HED-ICR*

Tumor	Days after intramuscular graft:								
	15	25	35	45	55	65	75	85	Still alive
6C3HED	3/15*	7/15	11/15	13/15	14/15	14/15	14/15	14/15	1
6C3HED-ICR	0/15	0/15	1/15	9/15	12/15	12/15	13/14	14/15	1

*The denominator shows the number of animals grafted, and the numerator the number of animals killed by the tumor.

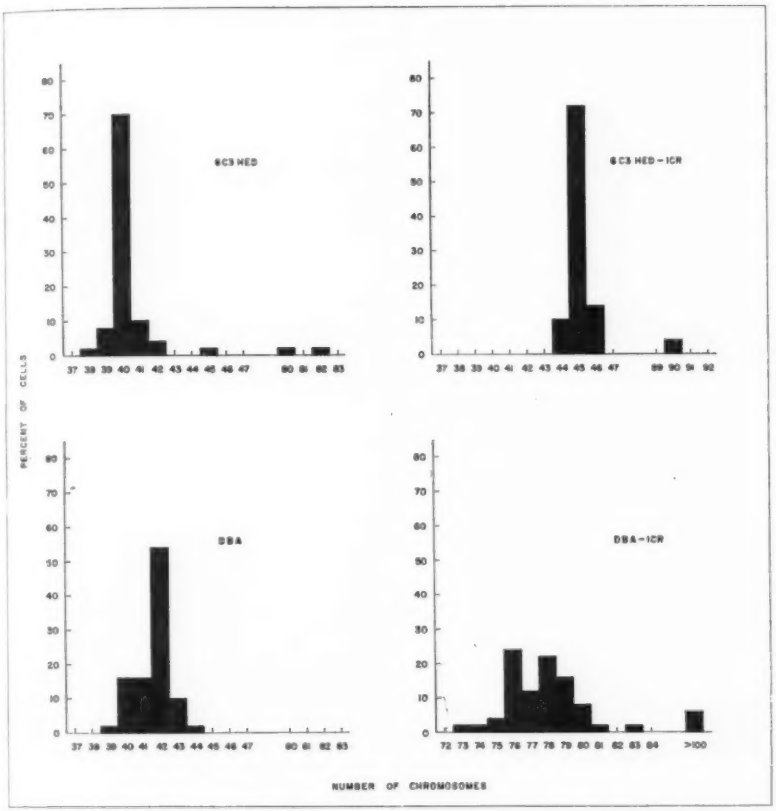
was found with 6C3HED-ICR only after 52 days. Similar results were obtained when pieces of liver, lung, and spleen were taken from C3H mice 6 days after an intraperitoneal inoculation of the original and adapted tumor. These data therefore show that both 6C3HED and 6C3HED-ICR metastasize in C3H, but that the 6C3HED metastases kill their secondary hosts more rapidly than those of 6C3HED-ICR.

Chromosomes of Original and Adapted Tumors

Previous studies on a variety of tumors have shown (*4, 7, 9, 16–19*) that nuclear changes in the form of differences in the chromosomes are related to tumor homotransplantability. It was therefore of interest to determine whether the same relationship exists between the original and adapted sublines. Koprowski *et al.* (*3*) had examined the chromosome numbers in the cells of these tumors, and in the present study the chromosome analysis was extended to include their morphological structure. This latter point was considered especially relevant, since a knowledge of the structure could be of considerable value in interpreting the mechanism of adaptation.

The chromosome analyses were all made on aceto-orcein squashes after pretreatment with hypotonic solution. The chromosome numbers in the original and adapted sublines are shown in text-figure 3, and each histogram is based on counts in 50 cells. These results confirm those of Koprowski *et al.* (*3*) in demonstrating a difference between the original and adapted sublines, and in showing that, as in all previously studied cases, the fundamental cells of the homotransplantable tumors have more than the diploid number, *i.e.*, duplicate chromosome material.

The study of chromosome structure has shown other differences which are especially clear in the comparison of 6C3HED and its adapted subline. In 6C3HED-ICR there are two very small chromosomes, one with a median centromere and one with a very wide secondary constriction (figs. 2–4). These types of chromosomes, and particularly those with a median centromere and a wide secondary constriction, have never been seen in the cells of 6C3HED (fig. 1) either in the present or previous studies with this tumor (*7, 19, 20*). These two latter types of chromosomes are also not found in cells of normal tissues of the mouse (*20, 21*). It can



TEXT-FIGURE 3.—Chromosome numbers in cells of the original and adapted tumors.

therefore be concluded that 6C3HED-ICR has new chromosome types that are not present in cells of the original tumor.

The analysis of DBA-ICR is more difficult than in 6C3HED-ICR, owing to the higher number of chromosomes. Although chromosomes with a median centromere or a characteristic secondary constriction were not found in this adapted subline, this does not exclude the possibility of smaller structure differences between this subline and the original DBA.

Discussion

Differences between Original and Adapted Tumors and the Mechanism of Homotransplantability

The loss of strain specificity is, from the immunogenetic point of view, one of the most significant properties involved in the adaptation of the original tumors. The concepts on the mechanism of homotransplantation are, therefore, of direct relevance to the objects of the present study. It has previously been pointed out (*8*) in relation to the general phenom-

enon of homotransplantability, that the loss of strain specificity cannot be interpreted by a loss of types of antigens, and that it is the antigenicity, not the number of antigens, that must be the focal point in this process. It was also argued, on theoretical grounds, that a loss of antigens must not necessarily lead to a decreased antigenicity. The analysis of the isoantigenic constitution of the adapted tumors has shown that 6C3HED-ICR has lost at least 2, and that DBA-ICR has lost at least 1 agglutinogen functional in the original lines of these tumors. But this antigenic simplification was not accompanied by a decrease in the antigenicity of the adapted sublines as measured in the intensity of the immune response elicited by tumor homografts. Neither the hemagglutinin production nor the cytotoxic immunity elicited by the adapted sublines were any weaker than those of the original tumors. It should also be noted that the loss of agglutinogens found in the ICR-adapted, and some other tumors (*5*, *6*) does not as yet show that there has been a loss of antigens that determine the cytotoxic immune response. There is, in fact, already evidence that these two types of antigens may be chemically quite different (*22*).

It has been shown that 6C3HED-ICR produces at least the same transplantation immunity as that of 6C3HED. This degree of immunity, however, does not destroy the homograft of 6C3HED-ICR, although it always causes a complete regression of the original 6C3HED homograft. It follows, therefore, that 6C3HED-ICR must possess some mechanism of resistance to the isoimmune response produced by itself. That it is the resistance that determines homotransplantability, and not a nonsusceptibility to immune response due to the lack of certain antigens, is indicated by the fact that the immunizing effects of a graft of MC1A can prevent the growth of a graft of 6C3HED-ICR.

In the previous immunological analysis of the MC1M and MC1A tumors (*8*), it had been suggested that the mechanism of tumor resistance to the isoimmune response is based on a high emission of antigens which can produce an extracellular neutralization of cytotoxic "antibodies." In these tumors a higher degree of resistance was found to be correlated with a higher immune response, as was theoretically to be expected on the assumption of a high emission of antigens.

Supposing that a similar mechanism of resistance exists in the adapted tumors, one may expect a similar correlation between the degree of resistance and degree of immunity. Since 6C3HED-ICR is more resistant than the original 6C3HED, one would expect it to be more antigenic than the original tumor, and this has indeed been experimentally demonstrated. On the other hand, since MC1A was found to be more antigenic than 6C3HED-ICR, one would tend to deduce a higher degree of resistance in MC1A than in 6C3HED-ICR, whereas the experiments seem to show the opposite result. The fact that 6C3HED-ICR has a higher degree of homotransplantability than MC1A does not necessarily indicate that it is the more resistant tumor, since in producing a lower immune response it can more easily overcome its own immunity.

It therefore seems possible that the homotransplantability of 6C3HED-ICR may be due to a somewhat different mechanism of resistance. This may be based on an intracellular rather than an extracellular neutralization of cytotoxic "antibodies." It is possible that cells of the adapted tumors may have a high rate of isoantigen synthesis and that these antigens may then be able to neutralize intracellularly cytotoxic "antibodies" without the latter causing a cytotoxic effect on the cells. So long as there is a certain excess of unaffected antigens, essential for the normal metabolism of the cell, there need then be no resulting cytotoxicity.

Another possible factor in the resistance may be connected with changes in the cell membrane. It has been shown that changes in the cell membrane, such as the lack of certain agglutinogens, have taken place in the adapted tumors. These may be accompanied by other changes which will favor the resistance of the cells to the cytotoxic factors.

Mechanism of the Adaptation

The major differences found between the original and adapted tumors include homotransplantability and resistance to an immune response, the antigenic components of the hemagglutinins, the chromosomes and the amounts of DNA and RNA (*3*), and susceptibility to certain viruses (*3*). It is thus of considerable interest to try and determine the mechanism by which the adaptations have taken place. There are three theoretical possibilities by which a tumor with new properties could have been produced in ICR hosts. 1) By the actual induction of a new and different tumor in ICR mice, 2) by the selection in the ICR hosts of cells with different properties that already exist as a small proportion of the original tumor, and 3) by changes in the original tumor cells produced as a direct result of the conditions under which the tumor was adapted.

The first possibility can be most readily excluded. The data on hemagglutinins and the transplantation tests have shown that the ICR sublines retain many properties of the original tumors. There is also so far no evidence that the adapted tumors have incorporated any of the antigenic components of the ICR hosts as would have been expected if these are now wholly or partly new tumors of ICR origin. The only data that may possibly indicate some sort of specific adaptation to the Swiss hosts are that the adapted tumors regrow very rapidly in Swiss mice after washing out the peritoneum. However, even this need not necessarily show any actual adaptation to the Swiss genotype during the original production of the ICR sublines. The adapted tumors have now been grown for many transplant generations in Swiss hosts, and there may have been after the original change some selection of cells that are more compatible metabolically with the Swiss strain and can rapidly regenerate a tumor from a small colony in these hosts. It has been found that 6C3HED-ICR has retained the agglutinogen K and DBA-ICR the agglutinogen D. These antigens are found in the strains of origin of these tumors, but not in Swiss (*4*). The Swiss hosts are therefore able to produce antibodies against the adapted tumors.

Due to the rapid regrowth of the tumor in Swiss mice no direct test of agglutinin production in these mice could be made. But it has been shown that cells of 6C3HED-ICR, when grown in Swiss mice for 11 days, have lost to a great extent their capacity to absorb *in vitro* anti-6C3HED-ICR agglutinins (table 8), thus indicating that the tumor cells are being coated with antibodies while growing in Swiss hosts.

Regarding the second possible mechanism, that of the selection of cells already existing in the original population, the loss of agglutinogen E by 6C3HED-ICR would have been of no adaptive value in Swiss mice, since this strain contains antigen E (*4*) and these mice would therefore not be able to produce antibodies against it. Koprowski *et al.* (*3*) have already drawn attention to the finding that the growth of 6C3HED in adult Swiss mice for 84 serial transplant generations produced no changes in transplantability (*4*). Even after further growth for 9 transplant generations in DBA/2 mice, it was only possible to select cells with a very low degree of homotransplantability in DBA/2 (*4*). If cells with radically different properties such as those found in the adapted 6C3HED-ICR were present in the original 6C3HED population, one would have expected them to be selected out during this very long period of growth in foreign hosts. Even if they were present as a very small proportion of the population, the existence of a large number of incompatible cells could have favored rather than prevented their growth in the foreign mouse (*23*).

The explanation of selection would also be difficult to reconcile with the chromosome data, which have shown the existence of morphologically distinct chromosomes in 6C3HED-ICR that have not been seen in the original 6C3HED or in cells of normal mouse tissues. The fact that the adaptation was carried out by grafting into fetuses or into hosts treated *in utero* with blood does not seem to weaken these points against the explanation of it being merely a question of selection.

In view of the above considerations, one has to consider the possibility that the changes in the ICR tumor cells were directly produced as a result of the conditions under which the adaptations were carried out. There seems to be a striking similarity between the adapted subline of 6C3HED produced by inoculation into ICR embryos, and the adapted sublines produced after grafting this tumor into ICR adults that had been inoculated *in utero* with C3H or F_1 blood. All the 3 sublines of 6C3HED-ICR are homotransplantable and appear to have the same modal chromosome number, and the same amounts of DNA and RNA. They are also all susceptible to oncolysis by Mengo, Bunyamwera, and West Nile, whereas the original 6C3HED is not susceptible to any of these viruses (*3*). If, as appears likely, the *in utero* inoculation of whole blood has produced only a partial tolerance, and if the same can be supposed for the *in utero* inoculations made with the tumor cells themselves, then it can reasonably be assumed that one is dealing with a common mechanism. The changes in the tumor cells could then be produced as a result of growth in a foreign host with a weak immunological response.

That such an explanation is feasible may be supported by the fact that

it has been possible to adapt a human ascites tumor to grow progressively without any further treatment in mice, after it had been passaged for a few transplant generations in mice treated with cortisone (*24*), *i.e.*, in hosts whose capacity to give an immune response had been considerably reduced. The changes in tumor homotransplantability that have been reported after growth of tumors in mice pretreated with lyophilized tissue (*25*, *26*) could also be explained in this way.

In addition to the human to mouse adaptation (*24*), Koprowski had been able to adapt a rat tumor to mice after *in utero* inoculations (*3*). It seems unlikely that the original human tumor already possessed cells capable of growing in mice, and this is also rather unlikely in the case of the rat to mouse adaptation. The fact that no changes had been observed with some solid mouse tumors (*27*) agrees with the failure to observe adaptation to mice with some solid human tumors (*24*). But rather than assume that this is due to a greater susceptibility of ascites tumors to selection (*27*), it seems probable that the metabolic conditions characteristic of the growth of ascites cells make them especially susceptible to the action of a directed change.

It may therefore reasonably be accepted that as a result of growth under a weak immunological response, there can be adaptation of the tumor cells to produce the resistance to antibody response found in the homotransplantable tumors. The adaptation to resistance could then bring with it the other properties, such as changes in chromosomes and nucleic acid and virus susceptibility, which are also found in other tumors that can give successful homografts.

Summary

A study has been made of the immunogenetic properties of the 6C3HED-ICR and DBA-ICR ascites tumors which have been adapted to give successful homografts as a result of inoculations into fetuses of ICR Swiss mice. This study has been mainly concerned with the analysis of the agglutinogen constitution, the agglutinin response to tumor homografts, the nature of the homograft reaction, the formation of metastases, and the morphology of the chromosomes.

It was shown that the adaptation of the 2 tumors had involved an antigenic simplification of their agglutinogens. 6C3HED-ICR had lost at least two, one of which was identified as E, and DBA-ICR had lost at least one agglutinogen possessed by the original tumors. It has also been found that 6C3HED-ICR had retained the agglutinogens C and K, and that DBA-ICR had retained the agglutinogens C and D.

This loss of antigens by the adapted tumors was not, however, accompanied by a decreased antigenicity as measured by the total agglutinin response to tumor homografts. It could further be shown for 6C3HED-ICR that the transplantation immunity elicited by its homografts was stronger than that elicited by the original 6C3HED, and that the adapted subline was susceptible to the cytotoxic factors produced by the homograft reaction.

The ability of 6C3HED-ICR to immunize a foreign host has also been confirmed by experiments on the transfer of immunity by activated lymph nodes.

It has been deduced from these results that the homotransplantability of the adapted tumors is due to a gain of resistance to the isoimmune response. It is suggested that the mechanism of resistance is due to a higher rate of isoantigen synthesis in the adapted tumors and that this can produce a neutralization of the cytotoxic "antibodies" elicited by the homograft reaction.

It has been found that the lethal effects of metastases of 6C3HED-ICR is less than that of the original 6C3HED, and that the adapted subline has some morphologically distinct chromosomes that have not been seen in the original tumor or in normal tissues of the mouse.

In a discussion on the various theoretical possibilities of the nature of the adaptation, evidence has been presented in support of the suggestion that the conditions of the adaptation have directly produced genetic changes in cells of the original tumors.

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PLATE 47

Photomicrographs of the chromosomes of the original 6C3HED, and the adapted 6C3HED-ICR and DBA-ICR ascites tumors. All aceto-orcein squashes after pretreatment with hypotonic solution. × 1,750

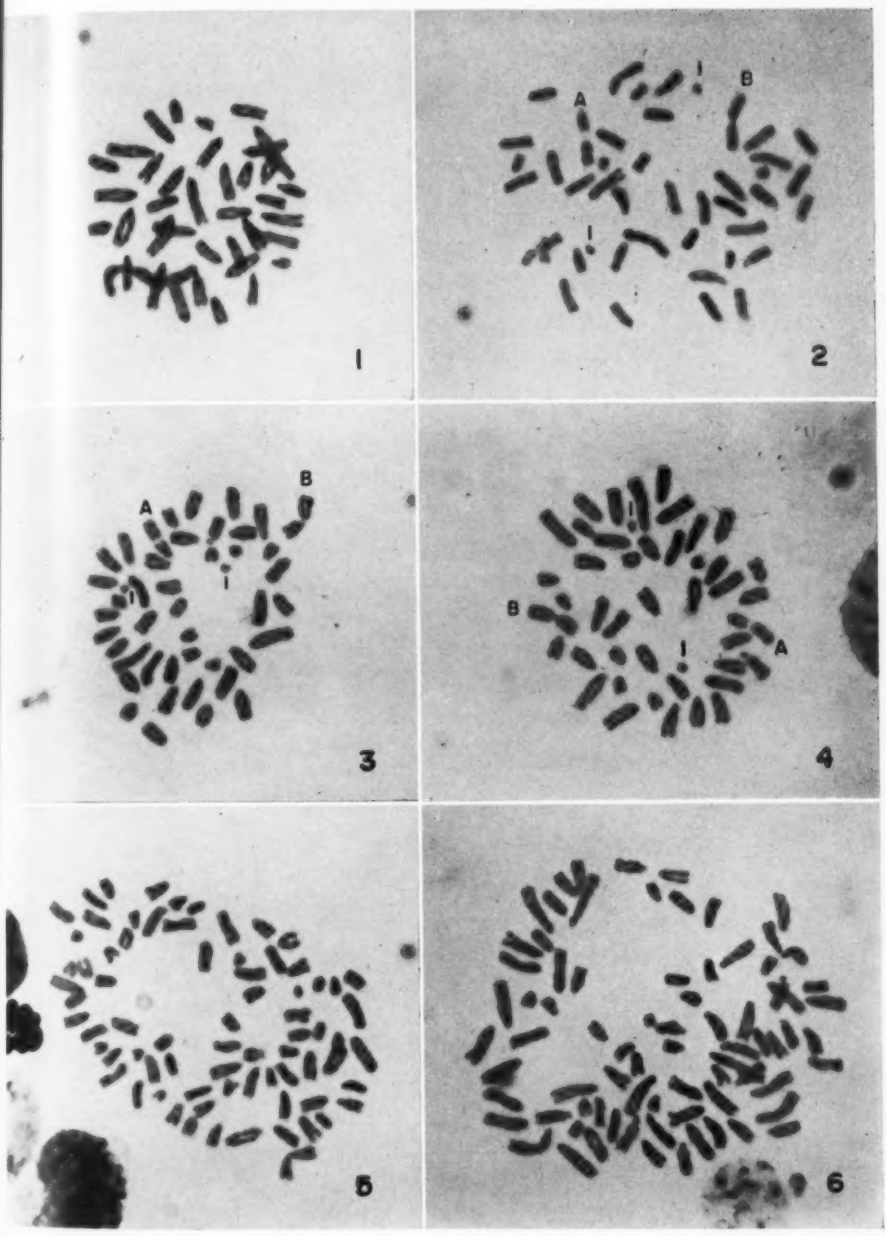
FIGURE 1.—6C3HED. Cell with 40 chromosomes.

FIGURE 2.—6C3HED-ICR. Cell with 44 chromosomes. I = very small chromosomes, A = chromosome with a wide secondary constriction, B = chromosome with a median centromere.

FIGURES 3 AND 4.—6C3HED-ICR. Cells with 45 chromosomes. I = very small chromosomes, A = chromosome with a wide secondary constriction, B = chromosome with a median centromere.

FIGURE 5.—DBA-ICR. Cell with 75 chromosomes.

FIGURE 6.—DBA-ICR. Cell with 77 chromosomes.


1
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B
2
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B
3
B
A
4
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6

Virus-like Particles in Hyperplastic Alveolar Nodules of the Mammary Gland of the C3H/He CRGL Mouse [1,2]

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We have recently been engaged in characterizing the typical hyperplastic alveolar nodule in the mammary gland of our C3H/He CRGL mice on the basis of various morphologic and physiologic criteria. The nodules are generally considered to be preneoplastic lesions (*1*, *2*). Grossly, histologically, and cytochemically, these hyperplastic nodules are not distinguishable from normal lobules of prelactating mammary tissue (*3*). Metabolically, they show an uptake of radiophosphate approaching that of prelactating tissue from mice in the last week of pregnancy (*4*). A difference between hyperplastic alveolar nodules and normal prelactating tissues, relative to the concentration of certain characteristic particles revealed by the electron microscope, is described herein.

Materials and Methods

Adult female mice from the C3H/He CRGL strain, infected with the mammary-tumor virus, were used as sources of nodular, normal prelactating, and tumorous mammary tissues. The following tissues were selected for study: 18 typical hyperplastic alveolar nodules (see *3*) from 12 animals, samples of normal prelactating gland from 14 animals, and samples of tumor from 6 animals. In addition, sections of normal prelactating tissue from 1 C57BL CRGL (virus-free) female and from 2 C3H/He females, which had been delivered by cesarian section and then fostered on virus-free C57BL mothers, were studied as controls.

Animals were killed by neck fracture. Tissues were dissected free of surrounding fat, and the prelactating and tumor tissues were cut into fragments measuring 1 to 2 mm.[3] All tissues were fixed within 15 minutes after death of the animal by immersion for 3 or 4 hours at 0° C. in Palade's osmium tetroxide fixative, buffered to *p*H 7.2 to 7.4. Material was dehydrated in ethanol and infiltrated with a mixture of 6 parts butyl methacrylate to 1 part methyl methacrylate containing 2 percent catalyst; the plastic was polymerized at 60° C.

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The most successful modifications of this procedure involved the use of phosphotungstic acid (approximately 24 $WO{\cdot}2H_3PO_4{\cdot}\ x\ H_2O$) as an added electron stain. For some specimens, osmic fixation was followed by treatment with 1 percent phosphotungstic acid adjusted to *p*H 5.4 in 0.1 *M* phthalate buffer for 18 hours at room temperature (*5*). In addition, 1 percent phosphotungstic acid was added to all alcohols used in dehydration. Other samples were fixed and dehydrated in the usual manner, except that the last 30-minute absolute ethanol bath contained 1 percent phosphotungstic acid. There appears to be no significant advantage for this tissue in the longer period of treatment with phosphotungstic acid. Most material treated with phosphotungstic acid by either method showed enhanced contrast in the electron beam. Membranes were particularly well stained. All sections were cut on a Porter-Blum microtome; these were mounted on collodion or carbon film and examined in a RCA EMU-2 microscope.[4]

Results

Preliminary cytologic examination of thin-sectioned prelactating, nodular, and tumorous tissues corroborates conclusions from light-microscope cytology and cytochemistry (*3*): No detectable difference appears in the structure or pattern of normal cell components in the nodule as compared with normal prelactating tissues of the same strain of mouse. Tumor tissue, in addition to showing much less intracellular and extracellular fat than the other two tissue types, frequently shows alterations in mitochondrial structure and possibly in the Golgi apparatus of the acinar cells. These observations, however, are incomplete and will not be discussed further at this time. Detailed examination of nodular and prelactating tissues was confined to those alveolar cells containing moderate amounts of osmiophilic lipide (*e.g.*, figs. 1 and 9).

Nodular tissue consistently shows in the cytoplasm and lumina appreciable numbers of spheroidal particles that cannot be identified with any normal cell constituent and that clearly resemble particles identified as viruses by other workers (*see* Discussion). These particles also appear abundantly in tumors. On the other hand, normal prelactating gland only infrequently shows small numbers of similar particles in the alveolar lumina.

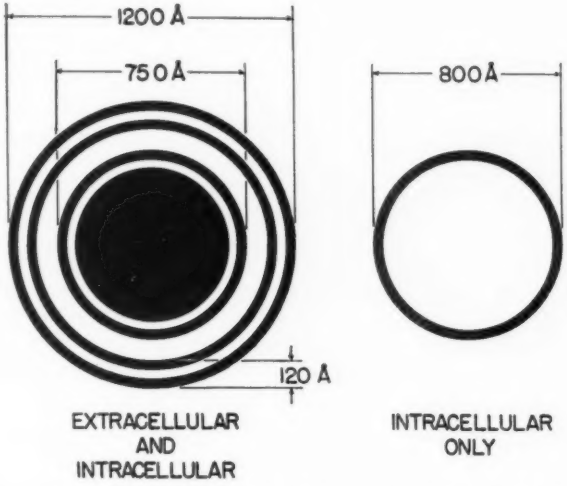
The particles are of two distinct types. A smaller particle occurs only within the cytoplasm of nodule and tumor cells. It varies from 650 to 990 Å in diameter, averaging about 800 Å. Although compression of the tissue during sectioning may cause both these and the larger particles to appear elliptical in section, both are probably spheroidal, and the longer diameter of their sectioned profiles is accepted as more nearly the true diameter (*6*). The small particle usually appears as a clear vesicle delimited by a strongly osmiophilic membrane, but occasionally its contents may be more or less electron-dense. These particles are illus-

4 For use of electron microscopes in their care, we are indebted to Dr. E. A. Steinhaus of the Department of Biological Controls, Dr. A. H. Gold of the Department of Plant Pathology, and Dr. T. L. Hayes of Donner Laboratory, all of the University of California; and to Mr. J. Murchio of Cutter Laboratories, Berkeley, Calif.

trated in the cytoplasm of a tumor cell in figure 2 and in a nodule cell in figure 8 (white arrows) where larger particles also are present.

The larger particles are abundantly present in the lumina of tumors and of nodules, and frequently in the cytoplasm (fig. 7) of cells from both types of tissue. They have a characteristic structure, best illustrated in figure 3. A double membrane, with a total thickness of about 120 Å, delimits the particle. Within this membrane is a clear zone about 80 Å wide; this in turn surrounds a central or slightly eccentric body averaging about 750 Å in diameter. This central body is limited by an osmiophilic membrane and contains a very dense region that varies in size. The over-all diameter of most of these encapsulated particles ranges from 850 to 1650 Å and averages about 1200 Å, but a few particles are even larger. Occasionally, one of the largest particles is seen to contain two inner densities (fig. 4). Both particles are illustrated diagrammatically in text-figure 1. Measurements were made on several hundred particles from both nodules and tumors, with no obvious differences between the two sources.

Spheroidal particles, of widely varying size, are present in large quantities in the lumina and cytoplasm of normal prelactating glands and of nodules. These particles are uniformly electron-dense, show no internal structure, and probably represent milk components. Figures 5 and 4, respectively, illustrate the contrast in appearance between these bodies in the lumen of a prelactating alveolus and the abundant characteristic particles seen extracellularly in a tumor. In figure 6, showing the lumen of a nodule, virus-like particles and secretion droplets are both present and clearly distinguishable.



TEXT-FIGURE 1.—Diagrams of vesicular and encapsulated particles from mammary nodule and tumor tissues of the C3H/He CRGL mouse, to show average dimensions.

In all three types of tissues, the cell surfaces bordering the lumina bear microvilli. These may be slender, fingerlike processes occasionally appearing on some normal prelactating cells (fig. 10), or they may be irregular, often branched, lobate protuberances (*e.g.*, figs. 1, 6, and 9). In fact, the entire apical border of the cells that do not show the large lipide vacuoles is frequently very irregular, with many invaginations from the lumen into the cytoplasm, as well as projections from the cytoplasm into the lumen. With the exception of some of the larger lobate processes, the microvilli are not seen to enclose particles. Instead, the virus-like particles, like the secretion droplets, may appear in depressions of the cell surface.

All 6 of the tumors examined contained these characteristic virus-like particles in the cytoplasm of occasional cells, and often in high concentration in the lumina (fig. 4). We have not yet examined enough samples of tumor tissue to be able to estimate the fraction of cells that contain virus-like particles, or to determine what part of the cytoplasm is most likely to enclose them.

Virus-like particles were found in all 18 nodules examined. In a few instances, examination of a nodule was cursory, being discontinued as soon as particles had been seen at any location within it. But whenever more than a few sections of any one nodule were studied, both intracellular and extracellular particles were readily demonstrable. It now appears probable that further study will show the presence of the particles in association with nearly every alveolar cell of a hyperplastic nodule. Both large and small intracellular particles are seen in greatest concentration near the luminal border of the cell, and do not show preferential association with any normal cell organelle.

Among the samples of normal prelactating gland from 14 mice, none contained detectable cytoplasmic particles of the characteristic structure described. Three samples showed some encapsulated particles in the alveolar lumina (fig. 10). None of the presumed virus-free glands (C57BL or C3Hd) showed similar particles (*see* addendum).

Discussion

Several other groups of workers investigating mammary tumors in various strains of mice have reported particles similar to those described above (*7–15*). These investigators found the particles in many tumors from mice known to carry the mammary-tumor virus, in some tumors from mice presumed to be free from the virus, and very rarely or not at all in normal mammary-gland tissue from virus-infected animals. Our present work demonstrates the presence of particles in the hyperplastic alveolar nodule, as well as in the tumor, of virus-infected C3H/He CRGL mice.

Most of the previous workers have seen particles of two types. The Dmochowski group (*14*, *15*) reported larger particles, similar to those found by us, ranging in size from 500 to 1500 Å; and small vesicular

particles averaging 650 Å in diameter. The larger particles in their micrographs occurred both within the cells and the lumina; the vesicular particles were all intracellular. Bang *et al.* (*10, 11*) found vesicular particles, about 700 Å in diameter, embedded in tumor-cell cytoplasm; and more complex particles, about 1300 to 1400 Å, clustered at the surface of the cells, in microvilli or in extracellular spaces. Bernhard and collaborators (*12, 13*) pictured small particles, 500 to 700 Å in diameter, abundant within the cytoplasm of tumor cells; and larger bodies, around 1000 Å in diameter, in extracellular sites. In each case, the small particle has an osmiophilic border and usually a low-density center. The large particle has a limiting membrane surrounding a clear zone, which in turn encloses a central or eccentric body of approximately the same dimensions as the small vesicular particle, but containing a very dense center. They (*13*) suggest that the small free particle develops into the central body of the larger one, and that the outer capsule of the larger particle is contributed by the host cytoplasm, as the particle is deposited extracellularly by being pinched off from the microvillus that contains it. A similar mechanism is suggested by Bang and his coworkers (*9–11*).

A corresponding relationship in size between the small, usually optically empty particle and the large complex particle is noted in our micrographs. However, we have no good examples showing the particles in microvilli, and the presence of abundant, fully formed, large particles within the cytoplasm of the host cell suggests that their development is completed before they emerge from the cell. An alternative possibility is that the intracytoplasmic large particles have entered the cell from the lumen where they are abundantly present, and that their outer capsule is lost during subsequent activity, to be formed anew upon later release from the cell. Thus, the encapsulated particle may be the infective form and the vesicular particle the reproductive form.

As has been frequently stated, the temptation to identify these particles with the mammary-tumor virus is almost irresistible. Certainly they occur in cells known to be virus-infected, where no other body appears as a likely candidate. Their similarity in structure and in postulated developmental sequence with other presumed virus particles is most striking (*e.g.*, see *16–19*). The mammary-tumor virus has thus far eluded all attempts to isolate and identify it (*15, 20*). Furthermore, the particles seen in sections have occurred in tumors for which biologic tests fail to reveal virus activity (*11, 13, 15*). However, circumstantial evidence in favor of their viral nature is so strong as to lead several workers to question their own negative bioassays. Dmochowski (*15*) has succeeded in demonstrating virus activity in the descendants of some mice whose supposedly virus-free tumors did indeed contain the suspect particles. Final proof must await the results of parallel biologic tests.

Granting the identity of the virus-like particles and the mammary-tumor agent, the presence of these particles within nodules and tumors does not establish a direct relationship between the agent and the neoplastic process. One cannot overlook the fact that transplantable mam-

mary tumors can arise in mice that yield a negative biologic test for the presence of mammary-tumor agent.

The presence of virus-like particles in all hyperplastic nodules examined makes reference to the nodule as a "virus lesion" factual, rather than speculative, as has been the situation until now (*2*). Examination of otherwise histologically indistinguishable prelactating tissue, from pregnant virus-bearing mice of the same strain, revealed only occasional instances of virus-like particles in our material. Bernhard *et al.* (*13*) reported one instance of intravillous vesicular particles in lactating tissue of a nontumorous mouse of their strain. The alveolar nodule, thus, is distinguishable from normal active tissue on the ultramicroscopic level, where it more closely resembles adenocarcinoma in its possession of appreciable numbers of the characteristic particles. Whether the nodules represent lesions induced by the virus or whether the nodules provide an environment favorable to multiplication of the virus which is then related to tumor induction, has not been determined.

Summary

Epithelia from normal prelactating tissue, hyperplastic alveolar nodules, and adenocarcinoma from the mammary glands of adult female C3H/He CRGL mice, infected with the mammary-tumor virus, were examined in the electron microscope. Special attention was paid to characteristic virus-like particles found in all 18 nodules, as well as in the 6 tumors, examined. Two kinds of particles were noted: 1) a smaller vesicular particle seen only in the cytoplasm, and 2) a larger encapsulated particle seen both in the cytoplasm and in the lumen. Only rare instances were encountered of particles in normal prelactating tissue (from 3 of 14 pregnant animals examined). These observations are interpreted as establishing the hyperplastic alveolar nodule as a "virus lesion." The nodule thus differs from its histologically indistinguishable normal counterpart (prelactating tissue) in the possession of large numbers of characteristic particles, and in this respect the nodule resembles the tumor.

Note Added in Proof

Since submission of this paper, we have examined many sections of prelactating tissue from 9 additional fostered C3H/He mice, in order to determine whether or not "viroid" particles are ever associated with mammary tissue in such mice. These mice were delivered by cesarian section and fostered on C57BL mothers and are presumed to be virus-free. In 2 of these 9 mice, a few particles were seen in the alveolar lumina, which were strongly suggestive of the extracellular (encapsulated) particles described in the text. The significance of this finding is not apparent; however, it should be noted that tumors arise occasionally in supposedly virus-free mice prepared as indicated above.

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All material fixed in Palade's solution and prepared by conventional methods unless otherwise specified. Scale line indicates 1μ unless otherwise labeled.

PLATE 48

FIGURE 1.—Low-power view of cells from a hyperplastic alveolar nodule. *Note* occasional microvillus arising from apical cell borders. These particular cells were selected because they do not show the typical huge lipide vacuoles, and thus allow clear illustration of organelles. In cytoplasm are nuclei (N), mitochondria (M), lipide droplets (L). Membranes (CM) are visible between cells in some areas. Characteristic virus-like particles are present in the lumen and near apices of cells but are not distinguishable at this magnification. × 13,500

FIGURE 2.—Cluster of vesicular particles with dense membranes and low-density interiors, near a mitochondrion (M) in the cytoplasm of a tumor cell. Additional particles appear at *lower left*. Association of vesicular particles with mitochondria was not seen as a rule. Treated by 18-hour immersion in aqueous phosphotungstic acid. × 39,600

FIGURE 3A AND B.—Two areas from the same section through lumen of a nodule, showing secretion droplets, some cell debris, and encapsulated virus-like particles. *Note* central density with surrounding single membrane, clear area, and outer double membrane. Treated by 30-minute immersion in alcoholic phosphotungstic acid. × 75,000

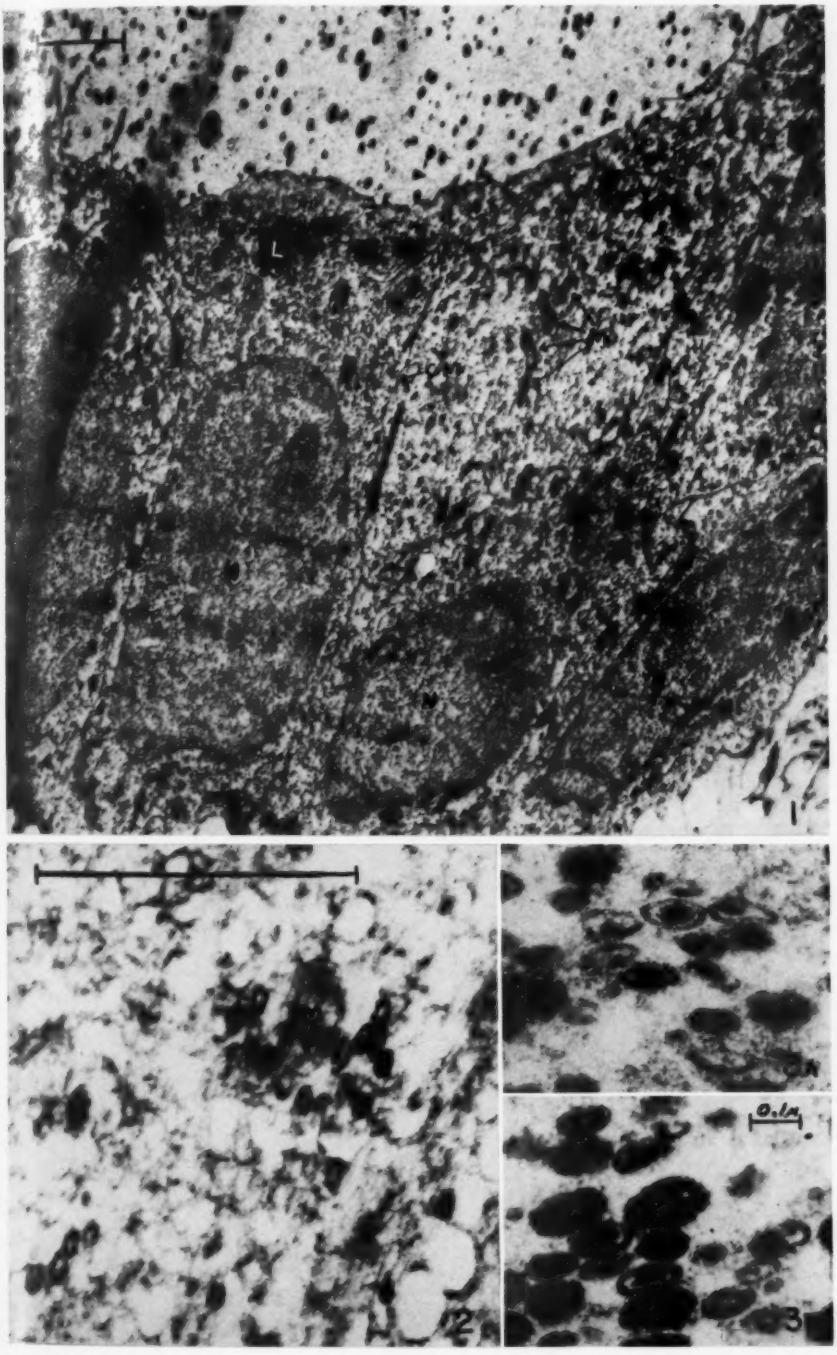
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PLATE 49

FIGURE 4.—Section from a tumor, showing lumen with cell border at *bottom*. *Note* high concentration of virus-like particles. One of them (*arrow*) contains two inner densities. × 39,600

FIGURE 5.—Section from normal prelactating gland from C3H/He mouse, showing lumen with cell border at bottom. *Note* high concentration of secretion droplets; some are embedded in cytoplasm, some in intracellular vacuoles. Treated by 30-minute immersion in alcoholic phosphotungstic acid. × 39,600

FIGURE 6.—Section from a nodule, showing lumen and cell borders at *bottom*. *Note* irregular cell border; mixture of cell debris, secretion droplets, and distinct virus-like particles in lumen; some secreted material in cytoplasm and in intracellular vacuoles; mitochondrion (M) in *lower right*. Treated by 30-minute immersion in alcoholic phosphotungstic acid. × 39,600

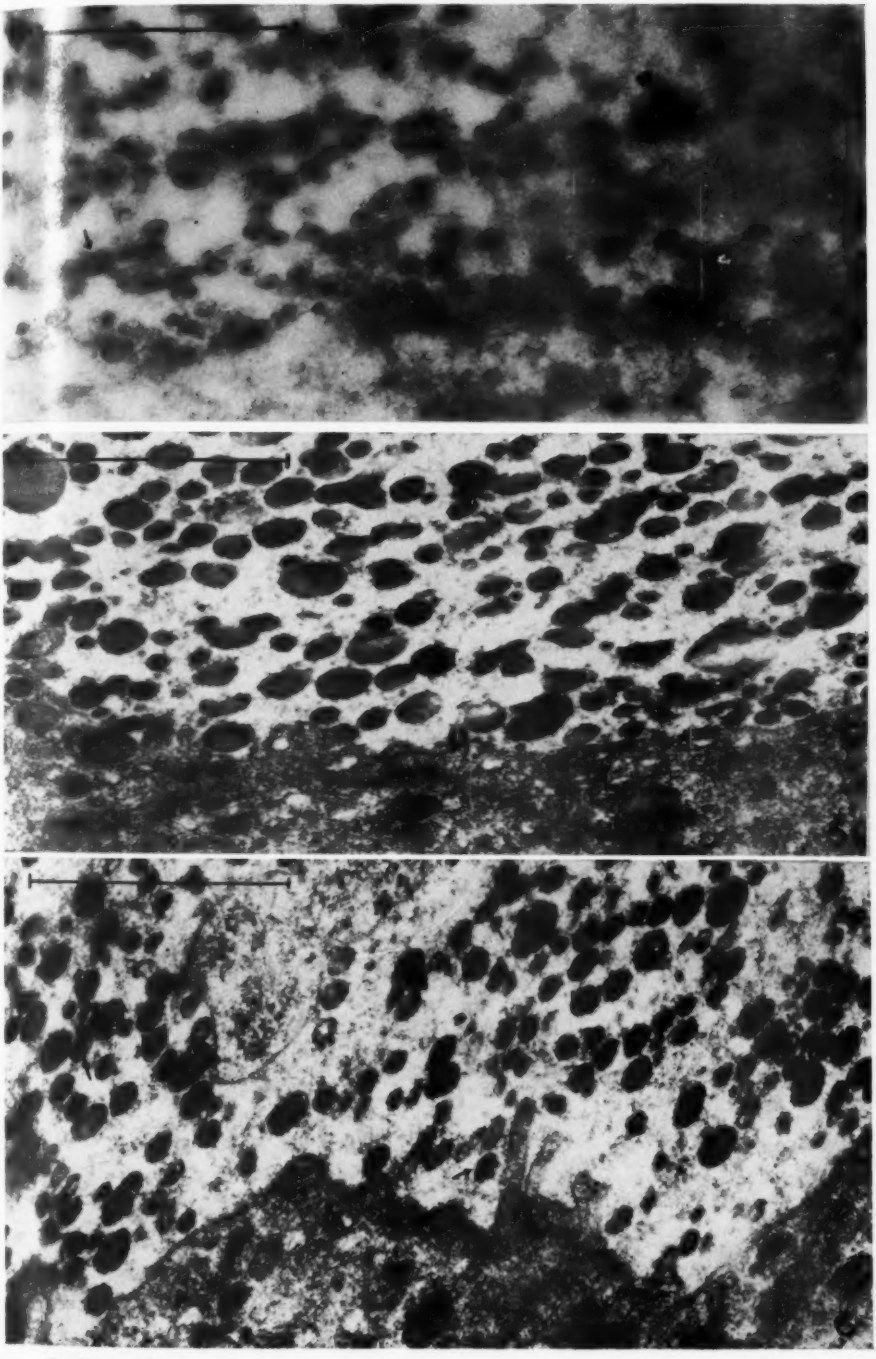


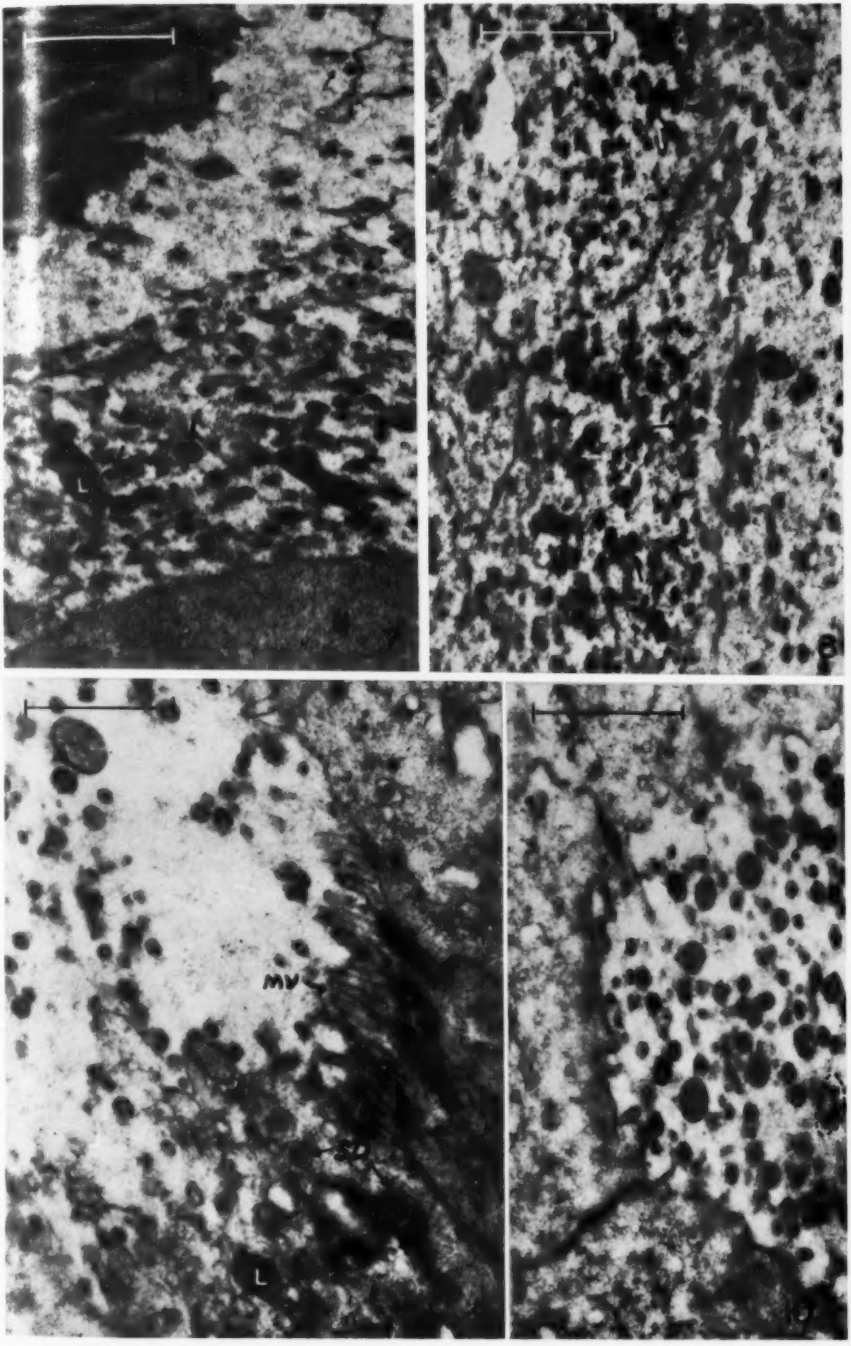
PLATE 50

FIGURE 7.—Section through small portion of nodule. Large lipide mass (L) in lumen at *upper left*, nucleus (N) at *bottom*, two intracytoplasmic lipide droplets (L). *Note* encapsulated virus-like particles in lumen and within cytoplasm (*arrows*). × 23,000

FIGURE 8.—Oblique section including parts of three cells of a nodule, lumen at *right*. Numerous encapsulated particles (*black arrows*) and vesicular particles (*white arrows*) in cytoplasm. × 19,200

FIGURE 9.—Section including parts of three cells bordering a lumen in a normal C3H/He prelactating gland. Microvilli on middle cell are very regular, cut almost longitudinally. Surface of lower cell is very irregular, with many microvilli cut obliquely. Secretion droplets (SD) in luminal invaginations or vacuoles within cell, as well as in lumen; intracellular lipide (L). × 23,000

FIGURE 10.—Section including parts of three cells bordering a lumen in a normal C3H/He prelactating gland. *Note* irregular cell surfaces, abundance of secretion droplets in lumen, and group of three encapsulated virus-like particles (*dotted circle*). × 23,000



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The Sex-Linked Histocompatibility Antigens [1]

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A very interesting exception to the previously accepted general rule that skin grafts within an inbred strain of mice are invariably successful was discovered by Eichwald and Silmser (*1*). They tested C57BL, A/Jax, and (A/Jax × C57BL)F_1 mice and found that there were many failures of the grafts when the donor was a male and the recipient a female, whereas there were almost no graft failures in all the other sex combinations. The male-to-female grafts were 100 percent unsuccessful in C57BL, C57BL to F_1, and A/Jax to F_1 mice, and approximately 50 percent unsuccessful in A/Jax to A/Jax mice. Prehn and Main (*2*) confirmed the male-to-female results for skin grafts in C57BL, but found no effect in BALB/c, DBA/2, or C3H mice. The effect has also been observed in C57BL mice by using grafts of thymic tissue (*3*). The immunological nature of the graft rejection has been shown by the existence of a second-set response (*4*).

The male-to-female effects that have been observed can be explained by the existence of sex-linked histocompatibility antigens. Although it seems most probable that the antigens concerned are linked to the Y chromosome (*4–6*), the existence of specific antigens due to a single X chromosome in the male cannot be completely ruled out (*7*). The published data also suggested that the different results obtained with various strains may be due to a threshold effect (*2, 4, 6*).

Our studies were undertaken in order to investigate:

1) The distribution of the sex-linked histocompatibility antigens in various strains.
2) The mechanism responsible for the rejection of the male-to-female grafts.
3) The possibility of utilizing these findings in order to separate sperm carrying the X from sperm carrying the Y chromosome.

Results and Discussion

In the present experiments, fitted pinch skin grafts, taken from the dorsal or lateral side of the body, were made according to the technique

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TABLE 1.—*Successful skin grafts within various strains of mice*

Donor→Recipient	C3H / C3H	BRS / BRS	C57BL / C57BL	A/Jax / A/Jax	A/Jax / F_1*	DBA/2 / DBA/2	BALB/c / BALB/c
♂→♀	0/21	0/21	0/16	0/12	0/8	0/5	0/4
♂→♂	8/8	9/10	7/7	7/7	†	5/5	4/4
♀→♀	10/10	7/7	8/8	6/6	6/6	4/4	†
♀→♂	10/10	8/8	†	†	†	†	7/7

*F_1 = A/Jax × C3H.
† Not tested.

of Billingham and Medawar (*8*). The grafts were fitted in such a way that hair growth on the graft would be in a direction opposite to that of the recipient so that it could be easily distinguished. The results with 6 inbred strains of mice and 1 F_1 combination are given in table 1; they show a clear effect in all the male-to-female transplants. The effect is therefore not specific to C57BL and A/Jax. The grafts were examined by external observation at varying intervals with an average of 10 days between inspections, and the survival times thus determined are shown in table 2. Although this rough way of measuring survival times obvivously produces inaccuracies and does not give exact figures, these results, and those of Eichwald *et al.* (*4*), show that the sex-linked antigens in all the strains tested are, in relation to the *H-2* system, weaker histocompatibility antigens like *H-3* and *H-1* (*9*, *10*). The analysis of this system therefore provides another approach to the study of the weaker types of antigens.

A point that was especially clear in BRS mice is that male-to-female grafts could be rejected even after the growth of new hair.

Second-set grafts were made at 42 to 80 days, depending on the strain, after the first graft. The results (table 2) show that there was invariably a second-set response, and in those cases in which it was tested, also a third-set response. This, therefore, also demonstrates the immunological nature of the graft rejection.

In order to determine the immunizing effects of various tissues, spleen, lymph nodes, and liver were inoculated intraperitoneally from males to females, and the results of one such experiment in which skin grafts were made 28 days after the inoculation are give in table 3. This shows that with this dosage and time interval there was an immunizing effect with both spleen and liver. These tissues, therefore, also contain the sex-linked antigens. This point is being further investigated in order to determine the best means of producing hyperimmune animals.

In regard to the *H-2* system it is known that tissue homografts elicit the production of hemagglutinins. We have therefore examined the production of this type of antibody in females immunized with male tissue, using the dextran-human serum method of Gorer and Mikulska (*11*). As is shown in table 4, no hemagglutinins were detected even after several immunizing doses with male tissue, so that also in this respect the sex-linked system which could be referred to as *H-4* behaves like *H-3* and *H-1* (*10*). It is therefore possible to obtain immunity to tissue transplants without the detection of hemagglutinins.

Another type of antibody, which has been found for the *H–2* system and which can be serologically tested, is the cytotoxic type of antibody described by Gorer and O'Gorman (*12*). The *in vitro* serological demonstration of these antibodies requires the addition of complement, but, although it has been found that these are not the antibodies responsible for the *in vivo* destruction of all tissue grafts (*13*), they can nevertheless serve as a valuable new indicator of certain antigenic differences. Using the method of Gorer and O'Gorman, we have tested for the presence of

TABLE 2.—*Breakdown time (in days) of ♂ to ♀ skin grafts*

Donor / Recipient	First set			Second set			Third set		
	No. of animals	Range	Mean	No. of animals	Range	Mean	No. of animals	Range	Mean
A/Jax / A/Jax	12	<23–87	55	9	<14–62	31	3	<12	<12
C3H / C3H	21	<22–71	48	19	<11–32	20	14	<11	<11
C57BL / C57BL	16	25–62	41	13	10–31	20	13	<12–15	15
BRS / BRS	21	<20–55	37	11	14–26	20	†	†	†
DBA/2 / DBA/2	5	13–55	34	5	<17–33	25	†	†	†
BALB/c / BALB/c	4	25–41	33	4	12–23	17	†	†	†
A/Jax / F_1*	8	26–36	31	8	<10	<10	†	†	†

*F_1 = A/Jax × C3H.
† Not tested.

TABLE 3.—*Effect of previous inoculation with various tissues on the number of successful C3H ♂-to-♀ skin grafts at 29 days*

With previous inoculation	
6 Lymph nodes	5/7
12 Lymph nodes	5/8
2 Spleens	2/10
0.2 cc. Liver	0/6
Without previous inoculation	
First-set skin graft	12/24
Second-set skin graft	0/19

these cytotoxic antibodies in the sex-linked system, in C57BL after 1, 2, and 3 skin grafts and in C3H after 3 inoculations of spleen and 1 skin graft. The tests were made with suspensions of male and female spleen cells, and no cytotoxic effect was detected in any of these tests. Although these results indicate that there are no such cytotoxins elicited in the sex-linked system, these experiments are being extended by the use of various methods of immunization, because it has been found that titers for these cytotoxic antibodies are lower than those for antibodies like hemagglutinins, so that in the case of tumor homografts positive results may sometimes be obtained only in really hyperimmune animals (*13*).

We are continuing these studies on serologically demonstrable antibodies in the sex-linked system, including *in vitro* tests on sperm followed by artificial insemination, both because of their interest in relation to the nature of the reaction responsible for the rejection of male-to-female grafts and because of their potential use as a reliable method for a directed sex determination.

TABLE 4.—*Tests for the presence of hemagglutinins after ♂-to-♀ immunization*

Strain	Type of treatment	Interval between treatments (*in days*)	Days since last treatment	Result
C57BL	3 Skin grafts	58, 31	12	*
C57BL	3 Skin grafts+1 inoculation of spleen and liver	58, 31, 14	10	*
A/Jax	4 Skin grafts	56, 44, 27	12	*
C3H	3 Skin grafts	58, 57	11	*
C3H	3 Inoculations of spleen+1 skin graft	31, 22, 21	34	*

*No hemagglutinins detected.

By utilizing the antigenic differences between male and female tissues, it should be possible to separate the X- from the Y-carrying sperm by producing an immune response either in the same or another species. Regarding the latter possibility, if, as seems likely, sex-linked antigenic differences exist in, *e.g.*, cattle and man, immunological tolerance (*14*) to mixed samples of female cattle or mixed samples of female human tissues can be produced in, *e.g.*, rabbits. The mixed female samples used for the production of tolerance would have to cover a range of genotypes within the species which is sufficient to eliminate the antibody response to all but the sex-linked antigens on subsequent immunization with male tissues. It may also be necessary to use mixed samples, covering a range of genotypes, for the immunization with male tissues, because there may be a variation in the sex-linked antigens like the antigenic variation that is known to exist for other histocompatibility systems. It should then be possible to separate the two types of sperm by producing in tolerant rabbits an immune response only to the sex-linked antigens of cattle or man. These possibilities are now being explored.

Summary

Male-to-female skin grafts were rejected 100 percent in 6 strains of mice (A/Jax, BALB/c, BRS, C3H, C57BL, and DBA/2) that were tested, whereas, as expected, skin grafts involving other sex combinations were successful. The male-to-female grafts could be rejected even after the growth of new hair. The immunological nature of the graft rejection has been confirmed by the existence of a second-set response in all the strains.

The presence of sex-linked histocompatibility antigens therefore seems to be a general phenomenon. In addition to skin, the antigens are present in other tissues such as liver and spleen.

The sex-linked antigens, in relation to the *H-2* system, seem to be weaker antigens like *H-3* and *H-1*. They do not elicit the production of detectable hemagglutinins, and also do not seem to elicit the type of cytotoxic antibodies that are detected by the addition of complement.

The antigenic differences between male and female tissues could be used in order to separate sperm carrying the X from sperm carrying the Y chromosome, and the possibilities of utilizing the sex-linked system for this purpose are discussed.

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Sex-Linked Rejection of Normal and Neoplastic Tissue. I. Distribution and Specificity [1,2]

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Female mice frequently reject skin grafts from males of their own strain (*1*, *2*). Several investigators have observed this phenomenon in certain strains of mice but not in others (*3*). Hirsch has presented evidence that thymic grafts of male C57BL mice are rejected by C57BL females (*4*). Feldman has demonstrated rejection of male lymph nodes of various strains by females of the same strain (*5*).

Rejection of male grafts by females of the same strain indicates that the graft carries an antigen absent in the host. This antigen is sex-linked and believed by some investigators to be determined by a histocompatibility gene located on the Y chromosome (*6*, *7*).

Early in our investigations concerning distribution of this antigen (male factor) in normal and neoplastic tissues of several strains of mice, the question of its strain specificity arose. It appeared that it was not a strain-specific antigen, but an antigen common to several strains. Experiments were then devised to test the validity of this observation. We will report data on the distribution and specificity of the male factor.

Materials and Methods

The mice used in the experiments were of the C57BL, A/Jax, C3H, ST, and BALB/c strains, and their reciprocal F_1 hybrids and backcrosses. The breeder colonies were originally obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and have been maintained by brother × sister matings. The mice were fed Purina pellets. Achromycin was administered once monthly with the drinking water.

Grafting of Skin

Our technique of skin grafting has been described (*8*). Each graft was placed adjacent to an autograft in the same bed. A graft was designated successful when it had failed to show signs of regression 5 months after

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grafting except as specified. The day on which grossly recognizable and irreversible regressive changes—scaling, discoloration, erosion, or ulceration—were first noted was designated as the rejection day. The first set of grafts was placed on the left, the second set on the right side of the body wall.

Grafting of Normal Tissue Other Than Skin

Grafts of various normal tissues, approximately 0.3 × 0.2 × 0.1 cm., were placed in bilateral subpannicular pockets. The animals were sacrificed 2 months after transfer, and the transplantation sites studied histologically. Successful grafts presented well-preserved portions of the transplanted tissue. Inability to locate the graft site or the presence of granulation tissue only was interpreted as failure.

Grafting of Neoplastic Tissue

Tumors transplanted serially for numerous generations are not suitable for a study of this kind. Information about the animal in which the tumor originated may not be reliable. The tumor may have undergone significant changes since the first transfer. Accelerated growth brought forth during several transfers may cover up the effect of relatively weak inhibiting factors. Also, most serially transplanted tumors appear to have arisen in female mice. The present investigation was, therefore, limited to spontaneous and chemically induced tumors. Tumors were induced by subpannicular injection of benzo[*a*]pyrene or 3-methylcholanthrene (Eastman, 1 mg. in 1 ml. of tricaprilyn) or by subpannicular implantation of crystals (methylcholanthrene only).

Tumor tissue was transplanted subpannicularly with a trocar. Tumor size was determined by weekly or twice weekly measurements, multiplying the largest diameter by the largest diameter at right angles to the former, disregarding depth.

Injection of Normal Tissues

Unless otherwise specified, 20 percent suspensions in normal saline were prepared by mincing the tissue in a hand-driven glass grinder; 0.5 ml. of the suspension was injected intraperitoneally, subpannicularly, or subcutaneously. Blood was injected intravenously in doses of 0.3 ml.

Hyperimmunization was accomplished by 10 intraperitoneal injections (twice weekly), using a 20 percent suspension of a mince of the kidneys, adrenals, lungs, spleen, heart, and about half the liver. The material for all but the first injection was stored at −20° C. Single injections of "normal cells" (referred to later) consisted of 0.5 ml. of the same mixture.

Observations

Skin

The data in table 1 show that the male factor can be demonstrated, with appropriate experimental design, in all male mice of all strains tested. The factor appears to differ in strength from strain to strain

TABLE 1.—*Transplantation of skin grafts*

Donor*	Recipient*	(Success/total)				Mean survival (*days*) of ♂ to ♀ failures (S. D.)	Range (*days*)
		♂ to ♂	♀ to ♀	♀ to ♂	♂ to ♀		
B	B	21/21	16/17	8/9	0/46	25.6 (±6.4)	16–45
B	BAF_1†	12/12	6/6	11/11	0/29	25.6 (±6.5)	17–46
B	BBC	—	—	—	0/6	20.8 (±4.7)	15–30
A	A	9/10	27/27	6/6	16/39	50.5 (±23.5)	18–109
A	BAF_1†	13/14	7/7	12/13	0/26	24.8 (± 4.3)	15–31
A	(A × ST)F_1†	—	—	—	7/8	96 (—)	—
A	ABC	5/5	4/4	6/6	5/14	29.5 (±16.1)	16–72
BAF_1	BAF_1†	24/24	12/12	8/8	0/9	30.2 (± 4.1)	25–38
ABC	BAF_1†	1/1	5/5	—	0/14	22.0 (± 6.2)	15–37
B/c	B/c	1/1	4/4	3/3	9/49	28.4 (±11.2)	12–51
B/c	(B/c × B)F_1†	—	2/2	—	0/9	16.8 (± 7.1)	9–27
(B/c × B)F_1	(B/c × B)F_1†	—	—	—	0/8	22.0 (± 3.8)	16–26
ST	ST	3/3	3/3	5/5	4/12	52.0 (±20.8)	33–90
ST	(ST × B)F_1†	2/2	—	—	0/14	20.6 (± 5.1)	15–28
ST	(ST × A)F_1†	—	—	—	0/7	63.1 (±52.8)	15–143
C3H	C3H	2/2	—	—	1/7	39.5 (±6.5)	28–49
C3H	(C3H × B)F_1†	5/5	—	—	0/17	23.5 (±3.6)	18–31

*B=C57BL; A=A/Jax; B/c=BALB/c; ST=Street; BBC=(C57BL × A/Jax)F_1† × C57BL; ABC=(A/Jax × C57BL)F_1† × A/Jax.
†Or their reciprocals.

and is most easily demonstrated when the recipients are females of certain F_1 hybrid combinations. Persistence of grafts in other sex combinations rules out other genetic differences as the cause of rejection.

All male grafts of all strains (and of their F_1 hybrids) were rejected when the recipients were F_1 hybrid females, provided one of the recipient's parents was of the C57BL strain. Rejection within the same strain was observed in all C57BL females, most BALB/c and C3H females, and in little more than half the A/Jax and ST females. Grafts of ST males were rejected by all (A/Jax × ST)F_1 females, while A/Jax male grafts were rejected by only 1 of 8 (A/Jax × ST)F_1 females. The time of rejection varied markedly and was as late as 143 days in an (A/Jax × ST)F_1 female.

Effect of Hormones

The data in table 2 show that castration (2 weeks prior to grafting) and administration of hormones (testosterone proprionate, 0.05 ml. subcutaneously, 2 weeks prior to grafting, repeated at grafting or 2 weeks thereafter) did not significantly influence the fate of male isografts in females. One of the female castrates failed to reject a male graft 3 months after grafting. She was then regrafted in order to rule out a technical error at the time of first grafting. The second grafting was technically unsuccessful and this mouse could not be evaluated. It is omitted from table 2. Survival of male grafts was somewhat shortened in females pretreated with testicular grafts, probably a second-set phenomenon.

TABLE 2.—*Effect of hormones on isologous rejection of male C57BL skin*

Recipients	Success/ total	Mean survival (*days*) (S. D.)
Untreated females	0/9	22.8 (±6.3)
Castrated females	0/17	22.2 (±6.1)
Testosterone-treated females	0/6	32.6 (±7.7) [Controls* : 27.5 (±7.1)]
Females with testicular grafts	0/4	19.3 (±0.5) (Controls: as above)
Untreated males	6/6	—
Castrated males	8/8	—

*Data of this table pooled from two experiments; only the first of the two experiments included a testosterone-treated group and a group with testicular grafts; therefore their controls are listed separately.

Effect of Pre- and Post-treatment on Females Rejecting and Females Failing to Reject Male Grafts

To verify the previously reported observation that second sets of male skin are rejected faster than first sets, additional groups of females received a second male graft after having rejected the first graft. The interval between first and second grafting ranged from 44 to 116 days. Thirteen C57BL females that had rejected the initial C57BL male graft after 25.7 (± 4.2)[4] days rejected a second graft after 13.2 (±3.8)[4] days.

[4] S. D.

The difference is statistically significant ($P<.004$). Three BAF_1[5] females that had rejected a first C57BL male graft after 21 (17–24) days rejected a second C57BL male graft after 13 (10–14) days. An A/Jax female that had rejected a first A/Jax male graft after 62 days rejected a second A/Jax male graft after 18 days.

The following paragraphs pertain to females that did not reject male grafts but received a second skin graft or other forms of post-treatment. In several mice post-treatment was initiated prior to the time that spontaneous late rejection could be excluded with certainty. These mice are excluded from table 1.

Four A/Jax females received a second A/Jax male graft after 103 days, in order to rule out a technical error at the time of first grafting and to elicit a second-set phenomenon manifesting itself as rejection of the second and probably also the first graft. (First sets never persisted in the presence of rejection of the second set and *vice versa*. Second male grafts on control males always persisted.) In two of the mice both isografts were rejected, in one of them, after 17 days, and, in the other, 53 days after the second grafting. Another mouse had failed to reject either graft by 60 days after the second grafting when it received, without effect, an intraperitoneal injection of male A/Jax normal cells in order to enhance any possible immune response. On the assumption that the antigen was not strain-specific, and was stronger in the C57BL than the A/Jax strain the mouse received, again without effect, two intraperitoneal injections of male C57BL normal cells. After 33 days a graft of male A/Jax skin was transferred to the subpannicular tissue of the host, on the assumption that a larger amount of antigen might thereby be administered and over a longer period of time. Thirteen days later, 159 days after the second, or 262 days after the first grafting, rejection of both isografts began. The fourth mouse of this group received the same post-treatments but had not rejected the grafts 14 months later when she died. One additional A/Jax female that had failed to reject a male A/Jax graft underwent the same post-treatment but did not receive a second (surface) graft. There was no rejection. The male graft was retained for over 7 months when the experiment was terminated. The male graft of another female A/Jax mouse underwent a crisis 61 days after grafting. Thereafter the graft looked healthy for a period of about 2 weeks when ultimate rejection of the graft began. A second graft was administered later, 103 days after the first, and was rejected after 21 days.

Four ABC[6] females that had failed to reject male A/Jax skin received an intraperitoneal injection of male A/Jax normal cells (three at 60 days and one 165 days after grafting). One of them died 37 days later with the graft intact. One mouse rejected the skin graft at 8 days, and another, 18 days later. The fourth mouse, previously injected on the 165th day, was again injected 199 days after grafting, without effect on the graft. After 221 days and, again, after 252 days, she received, without effect on the graft, an injection of male C57BL cells. A subpannicular graft of male A/Jax skin transferred after 284 days was also without effect 459 days after the original grafting when the mouse died of a spontaneous mammary cancer. Two additonal ABC females that had failed to reject received second grafts after 40 days. The isografts were affected neither by the transfer of lymph nodes (after 108 days) from another ABC female that had rejected male A/Jax skin nor by an intravenous injection of male A/Jax spleen cells (after 162 days). The experiment was terminated 223 days after the first grafting.

Four BALB/c females that had failed to reject the initial BALB/c male grafts received, 61 days after grafting, 0.5 ml. of a 20 percent lymph-node suspension prepared from BALB/c females that had rejected male BALB/c skin. The graft of one of them was rejected 17 days later. An intravenous suspension of male BALB/c spleen

[5] (C57BL × A/Jax)F_1, or reciprocals.

[6] (C57BL × A/Jax)F_1 × A/Jax, or reciprocals.

cells was administered to the remaining three mice 103 days after skin grafting; rejection of one graft occurred 49 days later, 152 days after skin grafting. During their period of observation, the remaining two grafts persisted 180 days. Two additional BALB/c females that had failed to reject received a second graft, one of them at 49 days, the other, 78 days after the first graft. Both first and second grafts on the latter mouse were retained for 132 days, after the second grafting, when she died. The other mouse received a subcutaneous transplant of immune lymph nodes (59 days after second graft) and an intravenous injection of male BALB/c spleen cells (113 days after second graft) without effect on the grafts, 270 days after the first grafting, when the experiment was terminated.

Three ST females that had failed to reject the first graft of ST male skin received a second graft, one of them at 74 days, and two, 89 days after the first. Rejection occurred in one of them 31 days after the second grafting. The other two mice retained their graft for the duration of the experiment (150 and 240 days after the second grafting); one of them had also received an intraperitoneal injection of male C57BL cells 44 days after the second graft.

Normal Tissues Other Than Skin

Survival of normal tissues other than skin was tested directly and indirectly.

Direct.—Attempts with heart and kidney tissue were abandoned because of widespread early necrosis of heart muscle fibers and renal tubular epithelium immediately following transfer even within the same strain and sex. This prevented easy differentiation of success from failure. Lung and salivary gland were more suitable.

The data in table 3 indicate that most C57BL females rejected lung tissue of C57BL males. There was little evidence of rejection of salivary gland. However, hyperimmunized females rejected grafts of salivary gland while almost all untreated females (and untreated males) retained them.

TABLE 3.—*Transplantation of isografts of C57BL normal tissue other than skin (success/total)*

Tissue	Recipient	♂ to ♂	♀ to ♀	♀ to ♂	♂ to ♀
Lung	C57BL	7/7	2/2	3/3	1/4
Salivary gland	C57BL	4/5	0/1	1/1	3/4

Effect of Hyperimmunization

Salivary gland	(C57BL × BALB/c)F_1:	♂ to untreated ♂: 6/7 ♂ to untreated ♀: 7/7 ♂ to treated ♀: 0/7

Indirect.—Transfer of various normal tissues was followed by transfer of tumor 58 (table 4) which is known to carry the male factor. The effect of pretreatment on growth of this tumor was recorded. Pretreatment of C57BL females and their F_1 hybrids with blood (0.3 ml.) or 20 percent suspensions of spleen or liver cells from male donors (0.5 ml.) caused inhibition of growth of tumor 58 (table 4) which indicated that the male factor was in the tissues used for pretreatment.

TABLE 4.—*Effect of pretreatment with normal male tissue other than skin on growth of tumor 58 in females of the C57BL strain and their F_1 hybrids*

Tissue used for pretreatment (*C57BL*)	Strain	Tumor growth (*Success/total*)	Mean size (*mm.²*) in 3rd week (*Range*)	Survival time (*days*) of positives (*S. D.*)
Blood 0.3 ml. intravenously	BAF_1	1/5	12. 5 (0–50)	46. 0 (—)
Spleen 0.5 ml. subcutaneously	C57BL	8/18	36. 5 (0–130)	43. 8 (±9. 4)
Liver 0.5 ml. subcutaneously	C57BL	14/18	167 (0–408)	37. 6 (±12. 3)
Untreated controls	BAF_1	3/3	231 (126–336)	35. 0 (—)

Neoplastic Tissue

The data in table 5 show the results obtained when 6 male and 6 female tumors were transferred to an equal number of males and females of the same strain, their F_1 hybrids, or related backcrosses. Seven of the 12 tumors were spontaneous and 5 were chemically induced. In no instance was progressive growth of a male tumor in all males associated with failure of the same tumor in all females. With most tumors arising in either sex, differences between male and female recipients as to incidence of growth, survival time, and tumor measurement appeared insignificant. A male fibrosarcoma (#74) that grew equally as well in females as in males was transferred to females that had rejected male isografts and to other females hyperimmunized against male tissue of the same strain; no inhibition of growth was observed. Another male fibrosarcoma (#87) grew in 10 of 12 males and in 7 of 11 females. However, hyperimmunization had no effect on the susceptibility of females to this tumor. A C57BL male lymphoma (#94) was transferable to 3 of 4 males, but to none of the 4 females; the tumor was lost on second transfer.

One of the tumors (tumor 58), a benzopyrene-induced fibrosarcoma in a C57BL male, has proved a useful tool for determining the distribution of the male factor in a variety of tissues. Growth failures were significantly more common in females than in males. The tumor also reached a larger size in males (mean: 300 mm.²) than in females (mean: 70 mm.²) on the 40th day after transplantation. However, the ranges within the male (0–690 mm.²) and the female (0–558 mm.²) groups were too wide to permit ready statistical evaluation. The difference in tumor growth between males and females has persisted in the subsequent 15 transfer generations. It was not affected by castration of males or females. There was a shortening of the survival time after the fifth transfer. Increased inhibition of tumor growth in female mice was noted following previous contact with tissues carrying the male factor (table 4). The tumor grew in only 1 of 7 hyperimmunized C57BL females while it grew in 6 of 7

TABLE 5.—*Transplantation of spontaneous and induced tumors to male and female recipients*

Tumor No.	Type*	Strain	Incidence of growth (*Success/total*)		Survival time (*days*) of positives (*S. D.*)	
			♂	♀	♂	♀
		Tumors Arising in *Females*				
1126	Mammary ca. (Sp)	A/Jax	4/4	4/4	76.0 (±6.0)	61.5 (±3.5)
41	Mammary ca. (Sp)	ST	14/14	14/14	57.6 (±20.5)	75.2 (±20.8)
35	Fibrosarcoma (BP)	C57BL	21/21	19/19	52.9 (±12.6)	54.4 (±20.4)
61	Mammary ca. (Sp)	ST	7/7	11/11	77.0 (±10.3)	66.0 (±22.4)
63	Mammary ca. (Sp)	BAF_1	20/20	16/16	112.0 (±18.0)	109.0 (±30.0)
72	Lymphoma (Sp)	C57BL	0/10	0/7	—	—
		Tumors Arising in *Males*				
68	Lymphoma (Sp)	BAF_1	0/10	0/10	—	—
74	Fibrosarcoma (MC)	C57BL	10/10	8/10	77.0 (±11.4)	88.0 (±18.6)
87	Fibrosarcoma (MC)	BALB/c	10/12	7/11	100.3 (±18.4)	92.1 (±31.8)
58	Fibrosarcoma (BP)	C57BL	27/30	12/26†	67.9 (±20.1)	63.0 (±31.8)
94	Lymphoma (Sp)	C57BL	3/4	0/4	—	—
35B	Fibrosarcoma (BP)	C57BL	20/20	17/20	33.6 (±12.6)	37.0 (±13.0)

*Sp = spontaneous; MC = methylcholanthrene; BP = benzopyrene.

†Significance of difference in tumor incidence between male and female recipients: $P = 0.0001$.

untreated female controls, 8 of 8 untreated male controls, and in 7 of 7 treated males.

Strain Specificity of Male Factor

In order to determine whether or not the male factor is strain-specific the following three experiments were carried out:

1) Skin grafts from 10 A/Jax males were transferred to BAF_1 males whose fathers were of the C57BL strain; skin grafts of 6 C57BL males were transferred to BAF_1 males whose fathers were of the A/Jax strain; skin grafts of 14 BAF_1 males were transferred to BAF_1 males, and care was taken that the father of the donor was of the C57BL strain when the father of the recipient was of the A/Jax strain, and *vice versa*. (In all three donor-host combinations the Y chromosome of the recipient was thereby derived from a strain different from that of the donor.) If the male factor of C57BL males had differed antigenically from that of A/Jax males, one would expect rejection of the grafts. Since no rejections were observed, it follows that the male factors of the C57BL and A/Jax strains do not differ antigenically.

TABLE 6.—*Specificity of male factor: Effect of pretreatment with spleen other than C57BL on growth of tumor 58 in female F_1 hybrids of C57BL mice, and saline controls*

Recipient	Pretreatment	Success/ total	Mean size ($mm.^2$) in 3rd week (*Range*)	Survival time (*days*) (*S. D.*)
(ST × C57BL)F_1*	ST, male	2/4	33 (0–80)	53. 5 (± 8. 5)
	Saline	5/5	456 (364–589)	39. 0 (±13. 6)
(C3H × C57BL)F_1*	C3H, male	2/5	24 (0–120)	43. 0 (±11. 0)
	Saline	5/5	560 (168–836)	30. 6 (± 4. 3)
C57BL	C57BL, female	5/5	276 (156–350)	34. 8 (±10. 7)
	Saline	5/5	287 (180–390)	30. 2 (± 3. 9)

*Or their reciprocals.

2) A group of BAF_1 females received skin grafts from A/Jax males, while another group of similar females received skin grafts from C57BL males. All grafts were rejected, the former after 23.4 days, and the latter after 21.3 days. Thirty-four days after the first grafting, both groups received a second graft of C57BL male skin. In both groups the second grafts were rejected faster than the first, though the second-set effect was more pronounced in mice that had first received a C57BL graft (11.5 days) than in mice that first received an A/Jax graft (13.0 days).

3) Female F_1 hybrids [(C57BL × C3H)F_1, (C57BL × ST)F_1] were pretreated with a single injection of splenic tissue from a male of their non-C57BL parent strain. Twenty-one days later, tumor 58 was injected into the pretreated hybrids and into untreated controls. The data in table 6 show that pretreatment with non-C57BL tissue significantly inhibited growth of tumor 58 indicating that the male factors of the strains in this experiment do not differ antigenically.

Discussion

Y-Linkage

Rejection of male grafts by females of the same strain would be explained by a histocompatibility gene located on the Y chromosome. However, Fox has presented evidence (*9*) that in *Drosophila* maleness and femaleness are associated with a variety of combinations of sex chromosomes. Females with Y chromosomes and males without Y chromosomes are not uncommon. These observations were more easily made in *Drosophila* because of the low number of chromosomes and relatively simple genetic structure of this species. While there is no evidence on which to base a similar supposition with regard to sex determination in mammals, it is difficult to refute the thought that a similar and, quite likely, much more complex situation may pertain, and that only because of this complexity in mammals no relevant observations have been made as yet. For this reason we have not used the term "Y-linked histocompatibility gene."

Manifestations of Male Factor

If one assumes that all cells of C57BL males (or the males of the other strains tested) carry the male factor, one wonders why not more than one of the male tumors tested showed evidence of it and why investigation of two of the male tumors revealed evidence that they lacked the male factor (being able to grow freely in hyperimmunized females). Did the method of investigation lack sufficient sensitivity to detect a weak factor, or was the factor lost in the chromosomal upheaval that occurred when the tumor arose?

Reactivity

Almost one half the A/Jax and ST females failed to reject male isografts of skin. Since male isografts were always rejected by the female F_1 progeny of these strains mated with C57BL mice, the question arose whether rejection by these F_1 hybrids was due to a contribution by the C57BL parent or to nonspecific "hybrid vigor." This question was answered by the observation that female F_1 hybrids derived from a mating of the strains in question with a non-C57BL mouse ("weak hybrid"), such as an (A/Jax $\times$ ST)F_1, did not reject male grafts more commonly than the pure strain females; in fact, (A/Jax $\times$ ST)F_1 females rarely rejected A/Jax male skin. This observation centers the attention on the contribution made by the C57BL component of the F_1 hybrid with respect to "reactivity" to an antigen proved to exist by the reported experiments.

It was also observed that female F_1 hybrids of the C57BL strain reacted less vigorously to such immunization procedures as pretreatment with one or multiple injections of various normal tissues, than pure C57BL females, as measured by inhibition of growth of tumor 58. This observation could be characterized, but not explained, as a "dilution effect."

The strain in which the male factor appeared the strongest, C57BL, also reacted most vigorously to it. It could therefore be argued that the strain differences observed with respect to the strength of the male factor are mere differences in reactivity. This would be in line with the observation that BAF_1 females rejected male A/Jax grafts as universally and as speedily as they rejected male C57BL grafts. Conceivably, both strength of the factor and strength of reactivity in a given strain may vary, and vary independently.

Weak Antigens

Tumor 58, of C57BL male origin, grew progressively in 48 percent of unrelated male backcrosses (ABC) indicating, as per conventional calculations, a single histocompatibility gene difference. Had the tumor been transferred to ABC mice of mixed sex the incidence of progressive growth very likely would have been lower since the tumor grows in only 50 to 90 percent of females. If the presence of the male factor is associated with a histocompatibility gene, it follows that the conventional way of calculating the number of histocompatibility genes has only limited applicability, being restricted to the determination of strong genes.

The male factor was detected with relative ease because it is presumably determined by a gene located on a chromosome with a distinctive phenotypic effect—maleness. It is quite likely that other and, possibly, countless similar weak factors are determined by genes scattered over other chromosomes. If these genes were part of the regular genetic composition of a strain (and not mutants) they would not be detectable by isografting because they would be shared by all members of a strain, male and female. If a weak antigen were determined by a gene on the X chromosome, it could be detected by grafting of male or female skin from a pure parent to male F_1 hybrids, and late rejection by those recipients whose X chromosome is derived from the strain other than the donor (*10*)—provided the antigen is strain-specific. If the genes were mutants they might be detectable by late rejection of skin grafts within the same strain; in tumor grafts they might be the cause of rejection of a second graft after surgical removal (or strangulation) of the first graft, or after administration of a subminimal number of cells.

Rejection of Normal Versus *Neoplastic Tissue*

It is often contended that skin grafts are more sensitive to, and, therefore, more readily rejected by, an immune response than tumor grafts. The reported experiments bear out this contention to a certain extent. All skin grafts of C57BL males were rejected, while the majority of grafts of tumor 58 grew progressively in C57BL females. There also is evidence to the contrary: rejection of skin grafts usually began during the fourth week after transplantation; by that time retardation or absence of growth of tumor 58 in C57BL females had been apparent for about 2 weeks. Conceivably this apparent contradiction is explained by the fact that rejection of tumors can express itself over a broader scale,

from barely noticeable retardation to full rejection. While full rejection of a skin graft is accomplished before full rejection of a tumor, retardation of a tumor becomes noticeable before a skin graft would show any regressive changes.

Distribution of Male Factor Within a Tumor-Cell Population

Growth inhibition instead of growth failure of tumor 58 in females was believed to be due to either multiple cell clones, some with and others without the male factor, resulting in growth of only a portion of the transferred cells and gradual immuno-selection of the cells without the male factor; or due to weakness of the male factor in all cells. In order to decide this question two lines of tumor 58 were carried through several generations, one in male and the other in female mice. At each transfer a group of mice of the opposite sex also was inoculated. If gradual selection of cells free of the male factor was taking place through filtering out of the factor-carrying cells, the difference of tumor growth in male and female mice would disappear in the female line and persist in the male line; also, transfer of the female-line tumor to females made resistant to male tissue by previous contact should be successful, while the transfer of the male-line tumor would be unsuccessful. That neither occurred indicates the absence of a selective process and the likelihood of a weak male factor in all tumor cells.

Summary

Normal and neoplastic tissues of various mouse strains were tested for the factor (male factor) that gives rise to the rejection of isografts of male skin by females. The male factor was found in males of all strains tested but the strength of rejection varied from strain to strain. It was strongest when donor and host were of the C57BL strain (100%) and weakest when donor and host were of the A/Jax strain (59%). However, rejection of male skin was universal in F_1 hybrids provided one of the parents was of the C57BL strain. The male factor was found in all tested normal tissues of C57BL males (lung, salivary gland, blood, spleen, and liver).

Only 1 of 6 male tumors was found to carry the male factor. The factor was evident by inhibited growth in female recipients. Inhibition could be accentuated by pretreatment.

Experiments utilizing reciprocal F_1 hybrids, second-set phenomena, and tumor 58 indicate that the male factor is not strain-specific.

The following aspects were discussed: Y-linkage; manifestations of the male factor; reactivity; weak antigens; rejection of normal *versus* neoplastic tissue; and distribution of the male factor within a tumor-cell population.

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An Attempt to Demonstrate a Y-Linked Histocompatibility Gene in the House Mouse [1,2]

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Eichwald, Silmser, and Wheeler (*1*) reported that the success of isologous skin grafts in C57BL mice was sex-influenced since females did not accept male skin grafts. These observations have been confirmed by Prehn and Main (*2*) and Hirsch (*3*). Eichwald *et al.* suggested that the influence of sex involved a histocompatibility gene located on the Y chromosome. Their evidence for this was based on the observation that C57BL females displayed the second-set phenomenon. Presumably this histocompatibility gene produces an isoantigen that is capable of immunizing an appropriate recipient. Hormonal experiments were also reported on a preliminary basis but were difficult to interpret.

A genetic interpretation, involving a histocompatibility factor on the Y chromosome, would be strengthened if it could be demonstrated that this factor was inherited by males whose father carried the gene and was absent in males whose father did not carry this factor. The experiments reported in this paper were initiated in order to test this possibility. They involved making appropriate skin grafts between hybrids of two strains, one that exhibited the influence of sex on skin grafting and the other that did not. Prehn and Main (*2*) reported that the effect of sex on isologous skin grafts was either weak or actually lacking in strain DBA/2 mice. This strain, therefore, was used in our study as the strain that lacked the factor. C57BL/6 mice served as the strain in which the factor was present. In addition, mice of the C57BL/10 strain were investigated to determine whether this strain, separated from the C57BL/6 subline prior to 1937, was similarly characterized by an influence of sex on skin grafting. Castrated C57BL/6 mice of both sexes received isologous male and female skin grafts in order to gain further information on the influence of hormonal factors on the observed sex difference.

Our genetic rationale was as follows: If DBA/2 males do not have the Y-histocompatibility gene in question, female hybrids (BL/6♀ × DBA/2♂) should accept male grafts of the same origin. On the other hand, hybrid females from the reciprocal cross (DBA/2♀ × BL/6♂)

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should reject grafts from males that received their Y chromosome from the BL/6 parent. In similar fashion DBF_1 hybrid males (DBA/2♀ × BL/6♂) should accept BDF_1 (BL/6♀ × DBA/2♂) hybrid male skin grafts, whereas the reverse situation should result in graft rejection.

Materials and Methods

The C57BL/6 and DBA/2 mice used in this study were obtained from the inbred nucleus of the Jackson Memorial Laboratory. The C57BL/10 mice were secured from Dr. George Snell's colony.

Animals were from 6 weeks to 4 months of age. Castrated hosts were gonadectomized at least 3 weeks prior to skin grafting. Split ear skin with the fat layer removed served as graft material. This was found to be satisfactory as it permitted easy recognition of the graft. The procedure for grafting was essentially that described by Bryant and Bernard (*4*). Animals were anesthetized with sodium pentobarbitol (Nembutal®) and each received a male and a female graft which were placed to the left and right of the mid-dorsal region. Bandages were removed 6 days after the operation and animals were examined at 5- to 7-day intervals thereafter. The condition of each graft was noted at each examination, and graft rejection was recorded when no sign of the graft was apparent. In all cases, except the DBA/2's which are still being followed, animals were killed and examined under a dissecting microscope 3 months after skin grafting. Castrated animals were autopsied at the termination of the experiment to insure the complete removal of the gonads.

Results

The results, which are summarized in tables 1 and 2, indicate that male C57BL/6 and C57BL/10 mice and reciprocal male hybrids of C57BL/6 × DBA/2 accept female skin, whereas females reject male skin. Similarly, castrated C57BL/6 mice respond to skin grafting the same as their non-castrated counterparts. With one exception male-to-male reciprocal hybrid grafts were accepted. The DBA/2 grafts, unfortunately, were not made until our hybrid data indicated that this strain must also carry the

TABLE 1.—*Incidence of successful skin grafts, intrastrain, castrates, and reciprocal hybrids (success/total)*

	♀ → ♀	♂ → ♂	♀ → ♂	♂ → ♀	$\overline{\text{MST}}$* ♂ → ♀	Range
C57BL/6 to C57BL/6	8/8	9/9	9/9	1/11	33†	16–85
C57BL/10 to C57BL/10	7/7	3/3	4/4	0/5	35	27–53
C57BL/6 to C57BL/6 (castrated)	15/15	11/11	13/13	0/12	29	16–41
(DBA/2♂ × BL/6♀) to (DBA/2♂ × BL/6♀)	8/8	5/5	5/5	0/10	45	16–54
(DBA/2♀ × BL/6♂) to (DBA/2♀ × BL/6♂)	8/8	7/7	8/8	0/7	36	16–63
DBA/2 to DBA/2	12/12	6/6	5/5	5/12	65†	52–105

*Mean survival time.

†MST of grafts that were sloughed.

TABLE 2.—*Incidence of successful ♂ to ♂ reciprocal hybrid grafts (success/total)*

♂(BL/6♀ × DBA/2♂)→♂(BL/6♀ × DBA/2♂)	5/5
♂(BL/6♀ × DBA/2♂)→♂(DBA/2♀ × BL/6♂)	5/6
♂(DBA/2♀ × BL/6♂)→♂(DBA/2♀ × BL/6♂)	7/7
♂(DBA/2♀ × BL/6♂)→♂(BL/6♀ × DBA/2♂)	5/5

sex factor. This was borne out by the fact that 7 of 12 DBA/2 females rejected male DBA/2 grafts. The remaining 5 grafts have been maintained for more than 130 days.

Discussion

Since it was found that the success of skin grafting was sex-influenced in the DBA/2 strain as well as in the C57BL/6 strain, it was not possible to obtain the genetic proof sought. This can only be accomplished when a strain that does not contain the factor or factors responsible for the influence of sex is incorporated into a hybrid study with a strain that does have the factor(s). We are continuing with the search for such a strain.

The finding that castration has no effect on the survival time of male-to-female grafts favors a genetic interpretation rather than a hormonal one. Moreover, if such an interpretation is valid, the same Y-linked factor is operating in both C57BL/6 and DBA/2 strains because grafts between reciprocal hybrid males were uniformly successful.

The mean survival time of male-to-female isologous and hybrid grafts appear to be the same except in the DBA/2's. The prolonged survival time in this strain, along with the persistence of 5 out of 12 male-to-female grafts, parallels the findings of Eichwald *et al.* (*1*) for the A/Jax strain. Here the acceptance of isologous skin grafts was sex-influenced to some extent. The success of grafts involving DBA/2-BL/6 reciprocal hybrids was like that reported by Eichwald *et al.* for A/Jax to F_1 (A/Jax × BL/6) hybrids. In these instances the influence of sex paralleled that in the C57BL strain. Eichwald *et al.* concluded that very likely these results indicated a relative weakness of the gene in the A/Jax strain. We offer the explanation that the genetic background determines the response of the host to the factor or factors. The genetic background of C57BL mice or their hybrids permits a stronger reaction to the responsible factor or factors than the genetic background of DBA/2 or A/Jax animals.

Summary

This study was initiated to gain further information on whether a Y-linked histocompatibility gene was responsible for the failure of isologous male-to-female skin grafts in certain strains. It was hoped that this could be achieved by making the appropriate skin grafts between hybrids of two strains, one that exhibited the influence of sex and one that did not. Since it was established that isologous skin grafts in both strains used in this study, C57BL/6 and DBA/2, were influenced by sex, it was not possible to prove the inheritance of a histocompatibility gene on the Y chromosome.

Castration had no effect on the survival time of male skin grafts to females in C57BL/6 mice.

If it is established that a Y-linked gene is responsible for the influence of sex, our data suggest that the same gene is present in both C57BL/6 and DBA/2 mice.

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Topical Antitumoral Effects of Cortisol, as Demonstrated by the "Double Granuloma-Pouch" Technique [1, 2]

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Many investigations have dealt with the effects of glucocorticoids upon the growth of malignant neoplasms in man and in experimental animals (for literature *see 1, 2*). The response to this kind of treatment depends largely upon the specific characteristics of the tumor studied, but various transplantable cancers are inhibited by glucocorticoids and stimulated by STH, somatotrophic hormone (*3, 4*).

In an earlier experimental series we attempted to answer the question whether the antitumoral effect of cortisol acetate (COL-Ac) is due to its direct topical effects or mediated indirectly through systemic metabolic actions. For this purpose we used the "granuloma-pouch" technique, in which a suspension of neoplastic tissue is injected into an artificially prepared subcutaneous air sac where it forms a rather regular, ellipsoid shell of tumor tissue. It was found that a given amount of COL-Ac, injected directly into the lumen of such a neoplastic pouch, exerts a much greater antitumoral effect than does the same amount administered subcutaneously at a distance from the neoplasm. This was thought to be a crucial experiment demonstrating the topical antitumoral effect of glucocorticoids (*5*). However, in the meantime, various observations made in our Institute have shown that the rates of absorption and destruction are not the same for hormones injected directly into the pouch as for those injected into normal subcutaneous tissue.

It is of fundamental importance in experimental cancer research to determine whether compounds act upon neoplasms (tumor cells and their stroma) directly or indirectly (through general metabolic effects). Hence, we attempted to modify the granuloma-pouch technique so that it can be used for the solution of this type of problem, irrespective of the rate of absorption or destruction of compounds which occurs at the site of injection. For this purpose, two tumor-bearing granuloma pouches are produced in the same animal, but the compound to be tested is injected into only one of them. Under these circumstances, the rates of absorption and topical inactivation of injected substances become immaterial,

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because the local effects of the compounds are compared with the distant actions upon an "internal control" neoplastic focus in the same individual. Yet the advantages of the granuloma-pouch technique are retained, in that the antitumoral material can be evenly applied to a comparatively regular, thin surface of neoplastic tissue, and variability due to loss of material such as occurs in the case of application to open surfaces is prevented.

It is the object of this communication to report upon experiments which indicate that if COL-Ac is thus applied to one of two Walker tumor-bearing granuloma pouches in the same animal the topical antitumoral effect of the hormone can be clearly demonstrated.

Materials and Methods

Twenty female Sprague-Dawley rats, with an average body weight of 136 gm. (range: 128/148 gm.), were subdivided into two equal groups, as indicated in table 1.

On the first day of the experiment *cranial granuloma pouches* were prepared, under ether anesthesia, in both groups. First, 15 ml. of air was introduced, through a 27-gauge injection needle, under the shaved skin of the back in the shoulder region; then, 0.5 ml. of an 0.25 percent solution of croton oil in sesame oil was injected into the resulting air sac, through the same needle. Forty-eight hours later, *caudal granuloma pouches* were made by injecting the same amount of air and croton oil in the lumbar region.

Single granuloma pouches are normally produced by subcutaneous injection of larger amounts of air at about the middle of the back, where the connective tissue is particularly loose, so that regularly shaped air sacs can readily be made. However, double granuloma pouches must be so spaced that they come to lie near the neck and in the lower lumbar region. In these locations the connective tissue is denser and emphysema-like, multiple, small air sacs tend to develop. To avoid this, it is important, before withdrawing the needle, to break any connective-tissue partitions that may have been formed with it. Furthermore, to prevent the coalescence of the two air sacs, it is best to permit the wall of the first established pouch to become dense and resistant (as a result of inflammation stimulated by the irritating croton oil) before making the second pouch. It is for this reason that, in the present experimental series, the cranial pouches were made 48 hours before the caudal ones.

TABLE 1.—*Topical antitumoral effects of cortisol, as demonstrated by the "double granuloma-pouch" technique*

Group	COL-Ac	Development of tumor tissue in pouches		Tumoral fluid (ml.) in pouches	
		Cranial	Caudal	Cranial	Caudal
I	In cranial pouch	+	+++	1.9±0.5	11±1.8
II	In caudal pouch	+++	Trace	30±1.9	0

Immediately after preparation of the caudal pouches, 0.5 ml. of a Walker-tumor suspension was injected into the cavities of both the cranial and the caudal air sacs (again through a 27-gauge needle). This suspension was made by mixing 1 part of the tissue of an 8-day-old Walker 256 carcinosarcoma (finely crushed in a glass homogenizer) with 4 parts of an 0.9 percent aqueous NaCl solution.

Immediately after the inoculation of the tumor material, 2 mg. of COL-Ac[3] microcrystals in 2 ml. of water was injected into the cranial pouches, while 2 ml. of water alone was introduced into the caudal pouches. The same amount of the COL-Ac suspension and of the water was then again introduced into the cranial and caudal pouches, respectively, on the 5th, 7th, 8th, 9th, and 10th days.

Differences in the development of the tumor might have resulted from two variables: (*a*) the air sacs were in two different locations, *i.e.*, the caudal and cranial regions; (*b*) the tissue suspension was injected into air sacs which were at different stages of development: one was freshly prepared, while in the other the croton oil had already produced a certain inflammatory reaction. To control these factors, we reversed the procedure in group II, keeping all other factors equal. That is, the cranial and caudal granuloma pouches were prepared in the same manner and at the same time as in group I, but whenever COL-Ac was injected into the cranial pouch and water into the caudal pouch in group I, we injected water into the cranial pouch and COL-Ac into the caudal pouch in group II.

The experiment was terminated after 12 days. The tumoral fluid was measured in a graduated cylinder and the amount of neoplastic growth in the pouch wall was assessed in terms of an arbitrary scale of 0 to +++. Accurate measurements of the weight of tumor tissue in the two pouches could not be made because the growth of neoplastic tissue was almost completely suppressed in the pouches that were directly treated with cortisol; consequently, the amount of cancer tissue could only be estimated by inspection with a magnifying glass and on histologic sections. On the other hand, the pouches that were not directly treated with cortisol contained enormous amounts of tumor tissue, so there could be no doubt about the topical antitumoral effect of the hormone.

Immediately after autopsy, specimens of all the pouch walls were fixed in susa solution for subsequent histologic examination of paraffin-embedded, hematoxylin-eosin-stained sections.

Results

Our results are summarized in table 1 and illustrated by figures 1 and 2.

It will be noted that neoplastic growth was almost completely suppressed and tumoral-fluid formation was greatly inhibited in the pouches that were topically treated with COL-Ac. The difference in the amount of fluid recovered from the cranial and caudal pouches, respectively, was highly significant ($P<0.01$) in both groups.

[3] The author gratefully acknowledges generous supplies of cortisol acetate (hydrocortisone acetate) from the Schering Corporation.

Histologic study showed an abundant growth of healthy Walker tumor tissue in the caudal pouches of group I and in the cranial pouches of group II. On the other hand, in the cranial pouches of group I and the caudal pouches of group II, that is, at the site of COL-Ac application, only scattered small islets of neoplastic cells were detectable.

The growth of neoplastic cells had not been completely prevented in any instance. However, the tumor nodules in the COL-Ac-treated cranial pouches of group I, though very little, were nevertheless noticeably larger than in the COL-Ac-treated caudal pouches of group II. We are tempted to ascribe this to the fact that in the former the malignant cell suspension was injected into an already inflamed territory, 2 days after the injection of croton oil, while in the latter, it was introduced into a focus of normal connective tissue simultaneously with the croton oil. Numerous earlier observations made in this laboratory suggest that a pre-existing inflammation greatly enhances the development of transplantable tumors in granuloma pouches and that the antitumoral effect of glucocorticoids is largely, if not entirely, due to their antiphlogistic properties. This view is further substantiated by the fact that under comparable conditions, the topical administration of deoxycorticosterone or cholesterol crystals failed to influence the growth of tumor cells in granuloma pouches. On the other hand, our observations do not permit differentiation between direct actions upon the malignant cells themselves and direct actions upon the stroma, particularly the blood vessels of the neoplasms. Earlier observations suggest that cortisol can exert some detrimental effect upon the neoplastic cells themselves under *in vitro* conditions where an action through the stroma is excluded (*6*).

Be that as it may, it is clear that, under our experimental conditions, COL-Ac exerts a topical antitumoral effect which can readily be detected by the double granuloma-pouch technique. It is suggested that this method may also be useful in the study of other antitumoral agents whenever topical and systemic tumor-inhibiting effects are to be compared.

Summary

If two "granuloma pouches" are produced in the same rat and cortisol acetate (COL-Ac) is injected into one of them, a marked antitumoral effect is manifest only in the pouch into which the hormone is introduced. It is concluded that glucocorticoids, such as COL-Ac, inhibit the growth of neoplastic tissue directly by virtue of local effects and not through the intermediary of systemic metabolic actions.

The "double granuloma-pouch" technique is recommended for the study of topical effects of various antitumoral agents. Its advantages are that the material to be tested can be evenly applied to a comparatively regular, thin surface of neoplastic tissue, and variability due to loss of material, such as occurs in the case of application to open surfaces, is prevented. Furthermore, in the usual tests, comparisons between topical and distant effects of antitumoral substances are complicated by the fact that compounds are not absorbed and locally destroyed at the same rate

after direct application to neoplastic tissue in one animal as after application at other sites in another animal. This difficulty is eliminated by the use of the double granuloma-pouch technique, because the local effects of the compounds injected into one pouch are compared with their distant actions upon another neoplastic focus in the same individual.

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PLATE 51

FIGURE 1.—Animals with Walker-tumor-bearing double granuloma pouches. Cortisol has been applied topically to the cranial pouch of the rat, *left*, and to the caudal pouch of the rat, *right*.

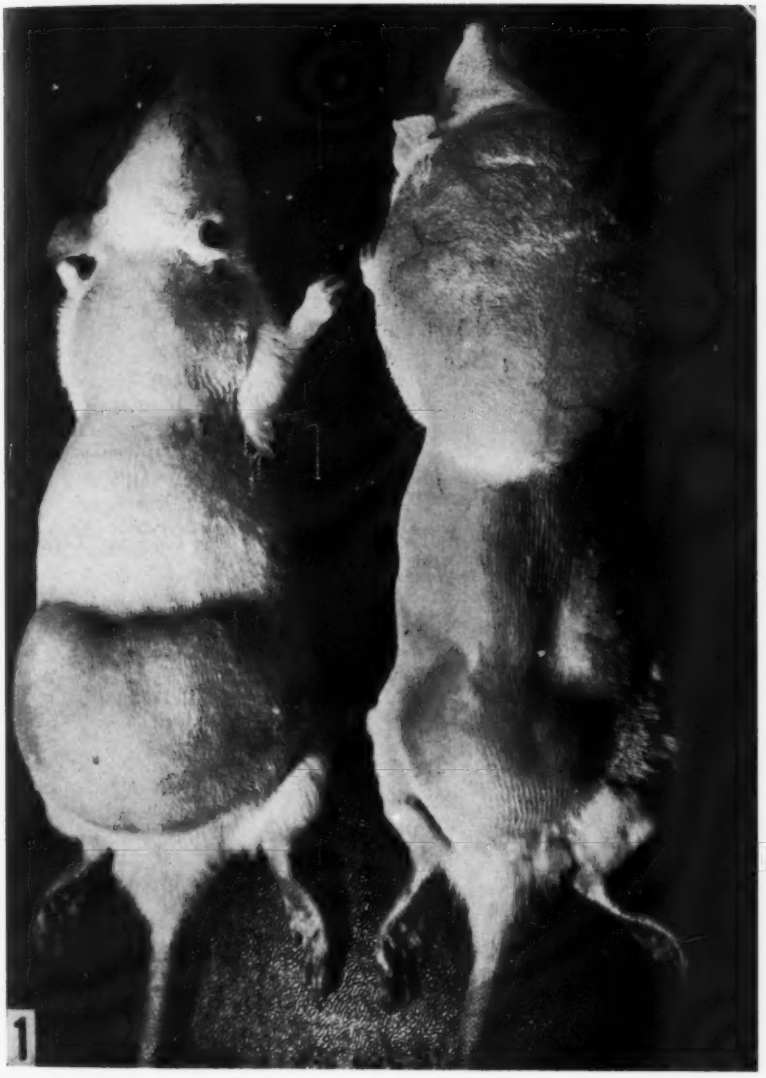

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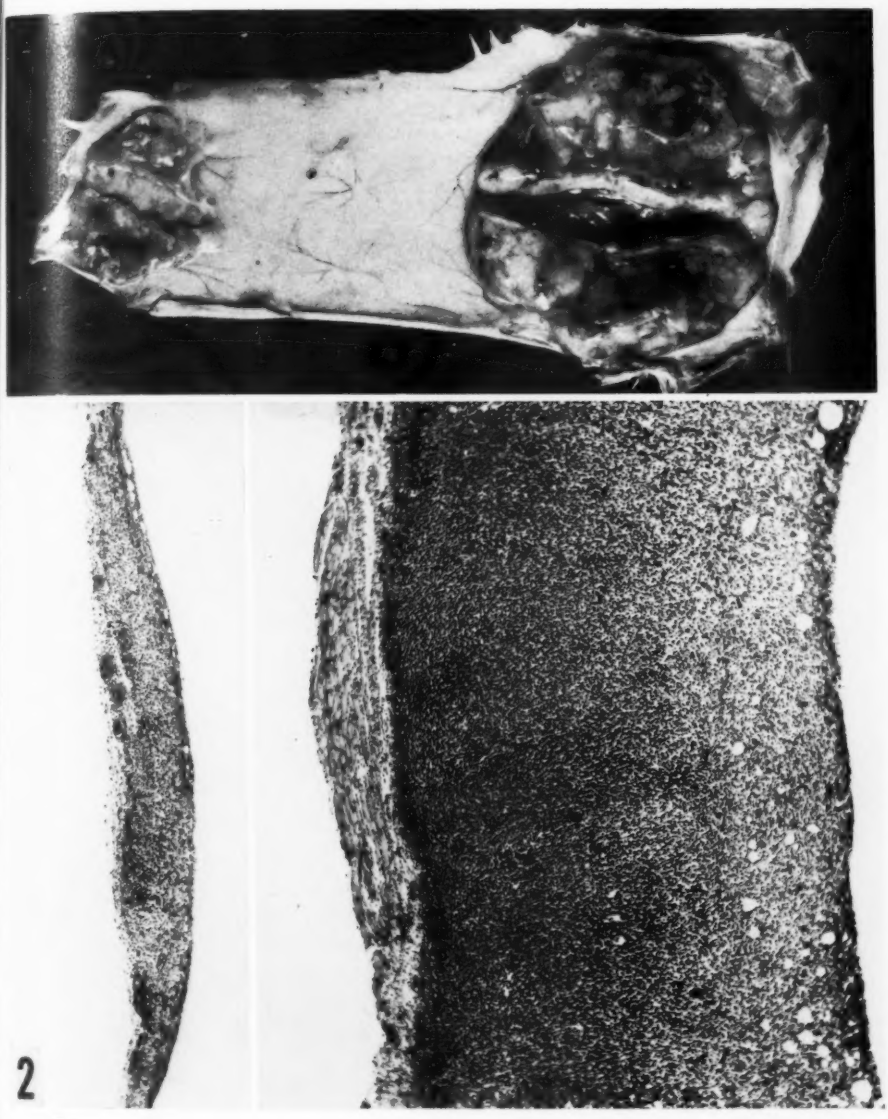
PLATE 52

FIGURE 2.—*Top:* ventral aspect of the dissected tumor-bearing granuloma pouches of a rat in which only the caudal pouch (*left*) was topically treated with cortisol. *Bottom:* histologic sections through the walls of the granuloma pouches shown above. Only a few islets of cancer cells (*dark area*) are detectable in the pouch topically treated with cortisol (*left*), while the wall of the other pouch is transformed into a thick, irregular shell consisting almost exclusively of neoplastic tissue. × 40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Relative Effects of Splenic and Bone-Marrow Cells on Lethally Irradiated Mice [1]

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Jacobson, the late Lorenz, and their coworkers (*1, 2*) reported that lethally X-irradiated mice can be afforded protection against death by lead shielding of the surgically exteriorized spleen of mice *during* irradiation or by an intravenous administration of bone-marrow cells *after* irradiation. It has now been firmly established that transplantation of erythrocyte, granulocyte, platelet, and thymocyte precursors takes place in treatment of lethally X-irradiated mice with rat bone-marrow cells (*3–11*).

Although radiation death of lethally irradiated mice can be prevented by treatment with foreign bone marrow, *i.e.*, from normal rats (RBM) or normal mice of another strain (HBM), many mice subsequently die, as first shown by Congdon and Lorenz (*12*) and later confirmed by others (*3, 13, 14*). These treated mice tend to recover from radiation effects in 2 weeks but then begin to undergo a severe reaction at about the 3rd or 4th week, reaching a critical period at about the 5th or 6th week. Many die during this period, but a significant number recover. Most of these survivors still carry the foreign transplant and thus are considered "tolerant" or "paralyzed."

Serum samples of 950 r-RBM[4] mice obtained at intervals of 7 to 65 days after treatment were analyzed with specific rat antimouse and mouse antirat sera (*15*) after preliminary experience with rabbit antisera (*3*). Serum samples beyond 65 days after treatment were not tested since the delayed foreign bone-marrow reaction took place within this period. No rat proteins were detected by the methods used, which indicates that if rat protein were present, it must be in concentration less than 12 μg. of protein-N per ml., the lower limit of sensitivity of the test. Since the number of serum samples was relatively limited, a more comprehensive study is now being undertaken. Pertinent data were also assimilated from immune-response studies (*7, 16*) and electrophoretic analysis of

[1] Received for publication September 20, 1957.

[2] Public Health Service Research Fellow of the National Cancer Institute.

[3] Operated by Union Carbide Nuclear Company for the U. S. Atomic Energy Commission.

[4] The groups of mice used in these experiments will be designated as follows, and the order of the symbols or numbers indicates the order of administration: 950 r-RBM, 950 r-HBM, 950 r-HSp, 950 r-IBM, 950 r-ISp. Isologous denotes intrastrain differences within a species; homologous denotes interstrain differences within a species.

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serum samples of irradiated mice treated with bone marrow (*15*, *17*). Based on these findings, the following hypothesis was formulated to explain the delayed foreign bone-marrow reaction. Lethal irradiation has caused a temporary curtailment of the immune mechanism of the host, and this permitted the transplantation of closely related foreign bone marrow. In time, however, there is a gradual recovery of the host antibody-producing cells that will react against the proliferating foreign transplant (*3*, *7*, *15*, *18*). Subsequent validity tests of this hypothesis showed that the *degree* and *time* of foreign bone-marrow reaction was dependent on the X-ray dose and that there was *no* increase in deaths with increase in foreign bone-marrow dose (*7*, *15*, *18*, *19*). Enhanced and increased incidence of death was obtained among 950 r-RBM mice preimmunized to rat nuclei antigens (*15*). These results would be expected on the basis of the above hypothesis.

Trentin (*20*) and Barnes *et al.* (*21*) submitted an alternative hypothesis; *i.e.*, the antibody-producing cells present in the transplanted bone-marrow tissue react against the host tissues and thereby cause the delayed reaction. In view of this expressed sentiment, it became necessary to investigate further the problem of the fate of lethally irradiated mice treated with foreign bone marrow.

Spleen of mammals (reviewed by McMaster, *22*, and Ruth *et al.*, *23*) and birds (*24*) is one of the few organs among the many implicated which, all investigators agree, is a site of antibody production. We felt, therefore, that information obtained from studies dealing with the relative effects of spleen and bone-marrow cells on the immune mechanism, as well as on survival patterns, of lethally irradiated mice should contribute to the solution of the problem.

Materials and Methods

Serology.—Twelve-week-old (C3H $\times$ 101)F_1 mice were divided according to sex, and randomized before irradiation. After a total-body irradiation of 950 r, they were caged in lots of 5 to 10. The X-irradiation factors have been described elsewhere (*18*). Within 4 hours after irradiation, each mouse received 1 ml. of 1 percent rat red blood cells (RBC) intraperitoneally and 12 $\times$ 10^6 normal isologous splenic (ISp) or bone marrow (IBM) nucleated cells intravenously. Spleen cell suspensions for intravenous injection were prepared by holding the spleen with forceps in a tilted petri dish containing a few milliliters of Tyrode's solution and teasing it apart with a narrow, pointed scalpel blade. The free cells and small particles that float into the Tyrode's solution were aspirated into a syringe and made into a cell suspension by passage through a fine needle. Large particles and pieces of spleen tissue were continuously pushed out of the Tyrode's solution with the point of the scalpel blade. Relatively little pulmonary embolism was encountered with this technique. Cell counts were made on the final cell suspension, which was diluted with Tyrode's to obtain the desired concentrations. These spleen cell suspensions differ from bone marrow in that they contain, in addition to blood-forming cells from

the red pulp, an undetermined proportion of lymphatic tissue cells from the white pulp. The bone marrow was prepared by the method described previously (*3*). The animals were allowed free access to food and water.

At intervals, up to 30 days after irradiation, lots of 10 mice were killed and the sera titrated individually. The methods used for the preparation of rat RBC for injection and titration of antibody were mentioned previously (*25*).

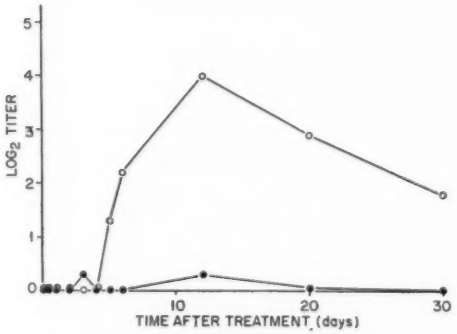
Survival effects.—The 5 experimental groups of mice were: 950 r, 950 r-IBM, 950 r-ISp, 950 r-HBM [$(L \times A)F_1$ bone marrow] and 950 r-HSp [$(L \times A)F_1$ spleen]. For controls, there were 2 groups of $(L \times A)F_1$ mice—950 r and 950 r-ISp. The bone-marrow dose was 12×10^6 nucleated cells, and the spleen dose 12×10^6 and 120×10^6 nucleated cells. The mice were divided according to sex, randomized before irradiation, given an intravenous injection of bone marrow or splenic cells within 4 hours after irradiation, and caged in lots of 10. Incidence of mortality was recorded through the 60th day after treatment.

The 30-day LD50 and LD100 X-ray doses for $(C3H \times 101)F_1$ mice were ~750 and ~875 r, respectively, and for $(L \times A)F_1$, ~600 and ~800 r, respectively.

Results

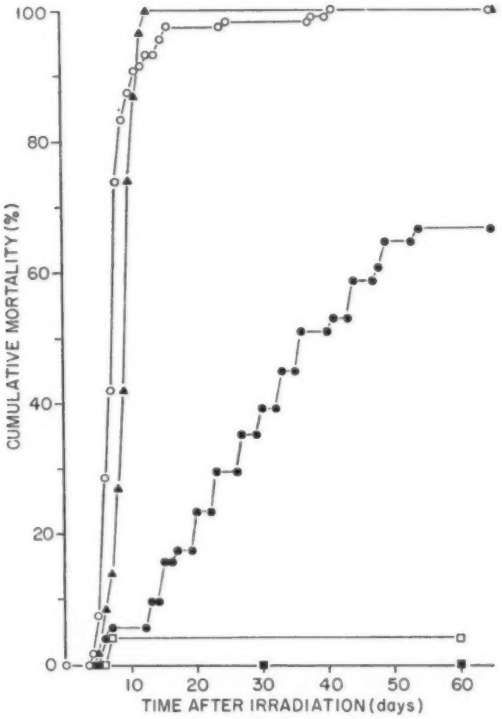
Antibody response.—The 950 r mice that received rat RBC simultaneously with IBM did not produce any detectable circulating antibody during the 30-day period of observation. In contrast, the 950 r mice that received rat RBC simultaneously with ISp responded. Circulating antibodies were first detected 5 days after treatment, and the peak titer was attained 12 days after treatment. The results are shown in text-figure 1.

Incidence of deaths.—All 93 of the 950 r mice died within 13 days after irradiation. Excellent protection, however, was afforded 950 r-IBM and 950 r-ISp mice. During the 60-day period of observation, none of the 22 950 r-IBM mice died; and only 1, of 24, 950 r-ISp died. Of the 51 950 r-HBM mice, 20 died in 30 days, and 14 died during the subsequent 30-



TEXT-FIGURE 1.—Antibody production in treated 950 r mice. Treatment: 950 r + rat RBC and 12×10^6 (a) ○ = splenic cells, (b) ● = bone-marrow cells. Ten mice per point.

to 60-day period. In contrast, all 119 of the 950 r-HSp mice died in 36 days, and 116 died within 16 days. These results are shown in text-figure 2.



TEXT-FIGURE 2.—Relative effects of spleen and bone marrow on 950 r mice (C3H × 101)F_1. ▲=950 r, 93 mice; □=950 r-ISp, 24 mice, 12 × 10^6 cells injected; ■=950 r-IBM, 22 mice, 12 × 10^6; ○=950 r-HSp, 119 mice, 12–120 × 10^6 cells injected; ●=950 r-HBM, 51 mice, 12 × 10^6 cells injected.

The results of the effect of dose of HSp on 950 r mice are shown in table 1. It can be seen that the initial onset of death is directly dependent on the dose of HSp cells. Of the 70 mice that received 12 × 10^6 cells, no deaths were recorded until the 6th day; but of the 49 that received 120 × 10^6 cells, 2 died on the 4th day and 7 on the 5th day.

None of the 950 r (L × A)F_1 mice survived, but 18, of the 25, 950 r-ISp (L × A)F_1 mice were protected.

Discussion

It has been established that autologous and isologous spleen can protect a lethally irradiated mouse (*1*, *26*). The results presented here, that isologous spleen is comparable to isologous bone marrow in its radiation-protective ability, are in accord with such findings. The efficiency of

TABLE 1.—*Effect of dose of* $(L \times A)F_1$ *splenic cells on 950 r* $(C3H \times 101)F_1$ *mice*

Dose of HSp $\times 10^6$ nucleated cells	Time after treatment (*days*)												
	1	2	3	4	5	6	7	8	9–10	11–12	13–14	15–16	36
	Cumulative death/total												
12	0/70	0/70	0/70	0/70	0/70	16/70	25/70	55/70	65/70	67/70	68/70	69/70	70/70
120	0/49	0/49	0/49	2/49	9/49	18/49	25/49	33/49	39/49	42/49	44/49	48/49	49/49

compatible spleen as a protective agent against radiation injury, when administered after irradiation, could be brought about by the granulopoietic cells and, possibly, by the platelet precursors *normally* found in spleens of mature mice, since (*a*) bone marrow (*2*), fetal liver (*27*, *28*), and compatible spleen (*1*, *26*) possess the protective agent; (*b*) thymus, reticulum sarcoma, lymphoma, lymph node, and monocytic cell suspensions lack it (*29*, *30*, *31*); and (*c*) the minimum requirement for protection against irradiation death is a temporary transplantation of granulopoietic cells after injection of bone-marrow cells in a lethally irradiated mouse (*15*). The importance of platelet precursors was not determined in the latter study.

Harris and coworkers (*32*) demonstrated that antibody-producing cells, when injected into sublethally irradiated homologous rabbits, synthesized antibodies, in spite of curtailment of the recipient's antibody-producing mechanism. This has been substantiated by Roberts and Dixon (*33*).

Thus, injection of a splenic cell suspension containing *both* the granulopoietic and antibody-producing cells into a lethally irradiated isologous mouse would be expected to be beneficial. However, if the lethally irradiated recipient is incompatible, the degree of injury would be dependent on the relative number of granulopoietic and antibody-producing cells if their relative rates of proliferation are assumed to be comparable. The granulopoietic cells will promote recovery of the recipient from radiation injury, whereas the antibody-producing cells will accentuate the deleterious effects of radiation by synthesizing antibodies against the recipient antigens. With this proposition in mind, we undertook the experiments reported here.

Two types of data are presented. The first demonstrates circulating antibodies in 950 r mice treated with ISp and the antigen. The 950 r mice treated with IBM and the antigen, on the other hand, did not synthesize a detectable amount of circulating antibodies during the 30-day period of observation. The dose of bone-marrow cells from normal donors was about 12 times the optimum amount necessary for protection against lethal irradiation. Only in preimmunized animals has it been reported that antibody-producing cells were detected in the bone marrow (*34*, *36*).

Based on these results, one would expect that injection of HSp into a lethally irradiated mouse would further accentuate the deleterious effects of radiation, but that HBM would operate in favor of recovery. Our second series of experiments confirmed this expectation. Only 10 percent of the 950 r-HBM mice died by the 10th day, 16 percent by the 15th day, 39 percent by the 30th day, and 67 percent by the 60th day. In contrast, 87 percent of the 950 r-HSp mice died by the 10th day, 96 percent by the 15th day, and 100 percent by the 36th day. Furthermore, by increasing the splenic cell dose tenfold (from 12×10^6 to 120×10^6 cells), we obtained earlier deaths. In contrast, it was shown previously that earlier deaths were not obtained when lethally irradiated mice were treated with an increasing amount of foreign bone marrow (*18*). In an attempt to induce skin-graft tolerance by injection of adult homologous (strains

C57BL and AU) splenic cells into newborn mice (strain A), Billingham and Brent (*37*), of Medawar's laboratory, obtained comparable results. These treated newborn mice appeared normal for the first week or two but died soon after. Schwartz *et al.* (*38*) reported a comparable killing effect with the injection of parent spleen into lethally irradiated F_1 hybrids; injection of bone marrow in a similar situation yielded an isologous type of protection. These results are in accord with our hypothesis discussed earlier concerning the delayed death patterns of lethally irradiated mice treated with foreign bone marrow.

Barnes and Loutit (*39*) reported that *infant* (4–7 days old) HSp can protect lethally irradiated mice for at least 30 days, in contrast to the early "killing" effect of adult HSp as reported here. This difference can be attributed to the lack of immunologically mature antibody-producing cells in infant spleens. Many of the lethally irradiated mice treated with *infant* HSp die at a late interval. It is very likely that the delayed death of these animals is the result of an *in vivo* antigen-antibody reaction involving *both* the recovered host antibody-producing cells and the "matured" donor antibody-producing cells.

Using chicken antisera, Weyzen and Vos (*40*) reported rat protein in serum samples obtained about 100 days after irradiation-RBM treatment. The rat protein can very well be derived from immature and mature white blood cells resulting from an immunological destruction. If this is so, then the highest concentration of rat protein in the serum would be expected to be present in the circulation when there is a severe foreign bone-marrow reaction. This possibility is now being investigated.

After we completed this work, our attention was called to a report by Uphoff (*41*) which dealt with the frequency of deaths after injection of bone marrow of parental strains into lethally X-irradiated F_1 hybrids. Data for the reciprocal test were not given, though it was stated that better protection was afforded by the F_1 hybrid marrow in lethally X-irradiated parental strains. Uphoff concluded that "evidence at present indicates that an immune response of the graft against the host, when the graft and host have different histocompatibility genotypes at the *H-2* locus, is responsible for the secondary phase of the irradiation syndrome." Exception to this statement can be found in the data. In the experiment where the F_1 is homozygous at the *H-2* locus and shares the same *H-2* allele with both parents (thus, antigenically compatible as far as the gene locus is concerned), the 90-day survival after treatment with parent 1 and 2 bone marrow was 70 and 76 percent, respectively. In 1 of the 3 groups where the F_1 does not share the same *H-2* allele with both parents, the 90-day survival after treatment with parent 1 and 2 bone marrow was 85 and 85 percent, respectively. Further comparison shows that (*a*) relative to the isologously treated F_1 mice, 90-day survival was 16 and 10 percent less, respectively, in the former group (*i.e.*, F_1 sharing the same *H-2* allele with both parents) and 15 and 15 percent less, respectively, in the latter group (F_1 not sharing the same *H-2* allele with both parents); and (*b*) the decrease in survivors between 14 and 90 days after treatment

was 18 and 9 percent, respectively, in the former group, and 15 and 12 percent respectively, in the latter group. In a comparable experiment, but with different strains of mice, Congdon of this laboratory (personal communication) and van Bekkum and Vos (*42*) found no delayed death patterns. These results indicate that loci other than *H-2* are involved; and especially important is an urgent consideration of X-irradiation dose, bone-marrow dose, and proper control experiments. For example, 900 r may be a 30-day LD100 dose for parent 1, parent 2, and F_1 mice; but relative to the 30-day LD50 dose, it may differ from 100 to 300 r, which is very important, especially in the supralethal dose range. With regard to the optimum marrow dose in homologous, as well as in heterologous experiments, it varies tremendously, depending on the host-donor genetic relationship. For example, 1 femur equivalent of strain X marrow may be sufficient to afford radiation protection of strain Y mouse, but a 3 femur equivalent of strain X marrow may be necessary to protect an irradiated strain Z mouse. Finally, because of the many unknown variables involved in marrow treatment of irradiated mice, it would be imperative that one carry out the effect of parent 1 marrow into parent 2 and *vice versa*, and F_1 marrow into parent 1 and into parent 2, when determining the effect of parent marrow in irradiated F_1 mice. Taking these points into consideration, if Uphoff's conclusion is shown to be the rule rather than the exception and if subsequent pathological and immunological investigations indicate that delayed deaths of these mice are attributable to an *in vivo* antigen-antibody reaction, then the problem of the foreign bone-marrow treatment of lethally irradiated recipients becomes even more complicated. One must then take into account the antibody-producing cells derived from the donor bone marrow, as well as those of the host.

Summary

1) Circulating antibodies were not detected among 950 r mice treated with isologous bone marrow and rat RBC but were detected among 950 r mice treated with isologous spleen and rat RBC.

2) Over 95 percent of the mice exposed to 950 r survived when treated immediately after irradiation with isologous bone marrow or splenic cells.

3) Only 39 percent of the 950 r-HBM mice died during the first 30 days after treatment, but 98 percent of the 950 r-HSp mice died. In the latter group of treated mice, earlier deaths were recorded when the dose of splenic cells was larger.

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Histochemical Comparison of Naphthol AS-Phosphates for the Demonstration of Phosphatases [1]

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Recently, several naphthol AS-phosphates have been described (*1*, *2*). In this paper, the synthesis, use, and histochemical comparison of a number of new phosphate esters of complex arylides of 2-hydroxy-3-naphthoic acid are described. Upon enzymatic hydrolysis these esters yield highly chromogenic, highly substantive naphthols, which result in very precise microscopic localizations. These substrates may be used with a variety of normal and neoplastic paraffin-embedded tissues as well as with cold formalin-fixed sections. The synthetic procedures and histochemical techniques were developed with primary consideration for their use in pathology laboratories.

Methods

Synthesis of Substrates

The substrates were synthesized by treating a suspension of the appropriate naphthol [4] (0.02 *M*, approximately 5 gm.) in tetrahydrofuran (50 to 70 ml.) with an excess of phosphorus oxychloride (4 ml.) and pyridine (2.0 ml.). The reaction mixture contained sodium chloride and sodium sulfate (approximately 1.0 gm. of each), which facilitated the synthesis. This would appear to confirm the work of Kraft *et al.*, who found that certain ions had a catalytic effect upon the yield of primary aryl phosphates (*3*). Most of the naphthols used are commercially available in 90 to 95 percent purity, which is satisfactory for synthesis. However, if recrystallized naphthol is used the sodium chloride and sodium sulfate as well as sodium lauryl sulfate and ethylenediaminetetraacetic acid EDTA (Versene®) (100 mg. of each) are used. The resultant naphthyl AS-phosphoryl dichloride may be transformed into the acid phosphate in a desiccator jar with potassium hydroxide as described by Freeman *et al.* (*4*). However, the acid phosphate was more conveni-

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[4] Naphthols furnished through the courtesy of the Pfister Chemical Works, Ridgefield, N. J., and Verona Dyestuffs, Union, N. J.

ently prepared by careful addition of water (as chips of ice) to the aforementioned tetrahydrofuran solution (or suspension of the dichloride) after the solvent has been evaporated under a jet of air. The reaction mass was then poured into 500 ml. of cold water, allowed to stand, and collected under suction. The disodium salt was prepared from the phosphate by the use of sodium methylate in methanol, or aqueous sodium carbonate. For routine laboratory use, isolation of the disodium salt is not necessary, and the following simplified procedure has proved useful. Fifty mg. of substrate are dissolved in 20 ml. (*N,N*-dimethylformamide) DMF. Twenty ml. water are added and enough 1 *M* sodium carbonate (usually 0.5 to 1.0 ml.) to adjust the *p*H to 8. Laboratory *p*H test paper is adequate to determine this point. Six hundred ml. distilled water are added and the volume is then brought up to 1 liter with 0.2 *M* tris buffer, *p*H 8.3 (for alkaline phosphatase), (24.2 gm. tris(hydroxymethyl)aminomethane, 100 ml. 1 *N* HCl, and water to make 1,000 ml.). Upon addition of buffer the solution will become opalescent. This stock solution is stable at room temperature for several months and available for immediate use. Its use will be subsequently described. For acid phosphatase 5.0 mg. of the acid phosphate are dissolved in 0.1 to 1.5 ml. DMF followed by distilled water and acetate buffer (*p*H 5.2).

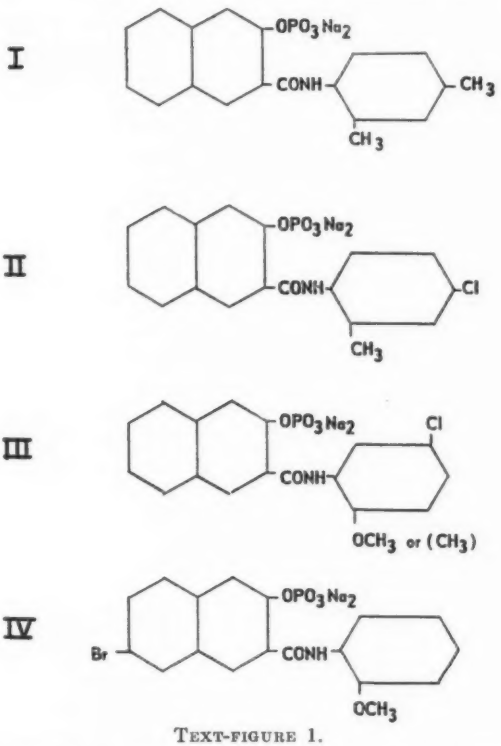
Phosphates of naphthols AS-MX (I), AS-TR (II), AS-CL (RCA) (III), and AS-BI (IV) were prepared and tested on tissue sections (text-fig. 1). In addition, phosphates of AS-D (the *o*-toluidide), AS-CL (the *o*-anisidide), AS-BS (the *m*-nitroanilide), AS-LT (the *o*-methyl-*p*-anisidide), and AS-ITR (the 5-chloro-2,4-dimethoxyanilide) were prepared but not tested thoroughly. These naphthyl phosphates may be prepared using the aforementioned technique at a cost of less than one dollar an ounce. Although naphthol AS-phosphate (*5*) was synthesized, it was found to be inferior to phosphates of the more complex arylides in localizations, as well as chromogenicity.

Preliminary work has indicated the feasibility of other methods for the preparation of AS-phosphates. The first procedure is based upon the use of polyphosphoric acid which has been used to phosphorylate alcohols (*6*). The AS-naphthol is heated in the presence of polyphosphoric acid upon a water bath. The barium or disodium salts may then be prepared. The second method utilizes a very smooth reaction, the coupling of an alcohol with an alkyl or aryl phosphite (*7*). The naphthol in tetrahydrofuran is treated with diethyl or dibenzyl phosphite in the presence of pyridine to form the resultant phosphate (*e.g.*, AS-D-diethyl phosphate).

$$(Et_2O)_2POC_{10}CONHC_6H_3CH_3$$

The alkyl or benzyl groups are removed by catalytic hydrogenation. The possibility of debenzylation with *N*-methylmorpholine warrants study.

With each substrate, approximately 5 mg. of substrate were dissolved in 0.5 to 1.0 ml. of *N,N*-dimethylformamide. Distilled water and tris



TEXT-FIGURE 1.

buffer (*p*H 8.3) were added to make a total volume of 50 ml. Forty mg. of diazonium salt were added, and the mixture filtered through rapid filter paper into a coplin jar. Fast Red RC, Red TR, Red Violet LB, Red RL, Violet B, Blue RR and Blue BBN salts were found to be useful as coupling components. The use of Red Violet LB salt (diazotized 5-benzamido-4-chloro-2-toluidine) has not been previously reported. This salt has a low decomposition rate, couples rapidly, and is highly chromogenic with the naphthols used. With Blue RR salt, beautiful shades of indigo were obtained (MX and TR-phosphates). Blue BBN salt also gave dark shades of blue. However, this diazonium salt is not as stable as some of the others at an alkaline *p*H.

The procedure of choice utilized the highly stable stock solution. (It should be noted that the stock solution is not hydrolyzed quite so rapidly as the acid phosphate.) Fifty ml. of stock solution were simply mixed with approximately 40 mg. of diazonium salt and the mixture filtered into a coplin jar. Incubation time for most paraffin-embedded tissues is 30 minutes. With formalin-fixed frozen sections very short incubations are possible (*e.g.*, 5 minutes for rat kidney). Although a *p*H around 8.3 was found to be useful for demonstrating alkaline phosphatase, the reaction may be run over a wide *p*H range and still give sharp localizations. Mg^{++}, Mn^{++}, or similar potential activators of phosphatase may

be used. In the case of acid phosphatase, Mn^{++} had a distinct activating effect. It should be noted, however, that the diazonium compounds used are zinc salts, and furthermore they may contain magnesium sulfate as a stabilizer.

For acid phosphatase incubation times varied from 30 minutes up to 6 or 8 hours, depending upon whether fresh-frozen or paraffin-embedded tissues were used. Stained sections were usually counterstained with hematoxylin, and mounted in glycerolgelatin. A polyvinyl pyrrolidone mounting medium is also useful (*8*).

Tissue Procedures

Frozen-dried, single and double embedded, acetone-fixed double-embedded (collodion and paraffin) tissues, and cold formalin-fixed material were used. Some raw-frozen sections were also tested to see if useful localizations could be obtained with unfixed tissues. The tissues included rat kidney, liver, spleen, pancreas, and bone (undecalcified 4-day-old hamster and mouse heads, and knee joints). Human neoplasms were studied. These consisted of lung carcinoma, carcinoma of the ovary, carcinoma of the colon, and a carcinoma of the liver. Several specimens of human breast and gingiva were also included, as was a transplanted mouse osteogenic sarcoma.

Observations

Very sharp localizations as well as intense shades of color were obtained with all substrates over a wide *p*H range. The localizations were much sharper than could be obtained with simpler unsubstituted naphthyl phosphates. All substrates were rapidly hydrolyzed, and an intense staining reaction was present in most active tissues in 15 to 30 minutes. Even raw-frozen sections gave good results. (With α-naphthyl phosphate, raw-frozen sections of kidney became surrounded by a cloud of diffused dye). The sharpest localizations were obtained with the frozen-dried and acetone-fixed double-embedded material, and with formalin-fixed frozen sections.

Naphthols AS-D, AS-OL, AS-BS, and AS-MX phosphates were most rapidly hydrolyzed. The most precise localizations were obtained with AS-MX, AS-TR, AS-CL, and AS-BI phosphates. Even though use of all substrates resulted in intense shades of color, the shades with AS-BS, AS-TR, AS-CL, and AS-MX were particularly striking. The combination of the MX or BS and Red Violet LB salt produced a brilliant crimson stain.

The distribution of activity was essentially similar to that observed with the older phosphatase methods (*9*). Therefore, not all tissues will be described in detail, and the findings, unless otherwise specified, relate to alkaline phosphatase. No nuclear stain was ever visible at any *p*H. The staining of arteriolar adventitial cells in various tissues was especially prominent (fig. 9). However, no reaction was observed in the elastica. The reaction of certain granulocytes in tissues was also pronounced because of their discrete staining.

Parenchymatous Tissues

The reaction in rat kidney shows a similar distribution to that obtained by other methods but is more sensitive and gives sharper localizations than previously observed. In the kidney tubules only staining of the brush border could be demonstrated (fig. 10). This is at variance with the observation of Deane *et al.* who used several simple naphthyl phosphates and observed staining throughout the tubular cytoplasm (*10*). With the use of carefully processed frozen-dried material this finding cannot be reproduced. In view of the more precise localizations obtainable with AS-phosphates, the possibility of diffusion artefacts should be considered in the use of simple naphthyl phosphates especially with raw-frozen sections. Dr. J. B. Longley informs me that the AS-phosphates clearly demonstrate the cell group of Goormaghtigh occurring at the angle between the efferent and afferent arterioles of the glomerulus (*11*). He has not been able to confirm this using the original Gomori method.

With the acid-phosphatase modification the brush border of the straight segment of the proximal tubules showed the greatest activity, as compared with the brush border of the convoluted portion. Moderate activity was seen in cytoplasm of the straight segment of the distal tubule (thick limb of Henle's loop, fig. 1).

The pancreas revealed a widespread activity of small capillaries. The connective tissue adjacent to ducts also stained. The islets of Langerhans showed activity of capillaries and also of peripheral areas which corresponded to sites of *alpha* cells (fig. 2). This appears to be similar to the distribution described by Dempsey *et al.* (*12*). However, in their work a 24-hour incubation was used with the pancreas, as contrasted with a 1-hour incubation in the present study.

The breast revealed a strong reaction of myo-epithelial cells adjacent to certain acini, as previously described by Huseby and Thomas (fig. 3) (*13*). One specimen showed activity of fat cells. Such activity has been previously observed by the author only with esterase methods.

Oral Tissues

Sections through oral and skin epithelium (hamster head) revealed activity of basement membrane (fig. 4). The activity was confined to the cytoplasm of connective-tissue cells and their fibrillar extensions. Sections of human gingiva exhibited activity of capillary endothelium and of scattered polymorphonuclear leukocytes. This reaction was very intense in inflamed gingiva.

Bone and Tooth Structure and Bone Tumor

Osteoblasts and connective-tissue cells associated with intramembranous bone formation exhibited a high activity. No activity was ever observed in bone or dentin matrices *per se*. However, fibers entering the periphery of the matrix were active. Although the distribution of activity was similar to that observed by other workers with older methods, the staining

was characterized by sharp localizations and absence of blurring or diffusion of dye to adjacent calcified matrices. Sections through the developing first and second molar teeth revealed activity in the stratum intermedium, odontoblastic layer, and adjacent pulp (fig. 5). The ameloblasts showed no activity, even with prolonged incubation periods. Myeloid elements of bone marrow stained intensely (fig. 6). The MX-phosphate (*p*H 8.5) was found to be useful in the staining of blood smears using the modification of Kaplow (*14*).

The osteogenic sarcoma, a transplanted mouse tumor, contained small foci of osteoid and calcified spicules. The tumor cells usually had large nuclei and a relatively small amount of cytoplasm, which stained intensely (fig. 11). This peripheral cytoplasmic reaction gave the appearance of a syncytium.

Soft Tissue Tumors

A carcinoma of the lung (adenocarcinoma) in a 55-year-old man was composed of sheets of atypical polygonal-shaped cells and areas of columnar differentiation adjacent to these cells. Phosphatase activity was observed in certain individual cells as well as groups of cells. In addition, the zone of columnar differentiation stained intensely (fig. 7).

The ovarian adenocarcinoma from a 60-year-old woman exhibited clusters of tumor cells arranged in a loose stroma. The cytoplasmic phosphatase staining was superficially similar to that observed in the osteogenic sarcoma (fig. 8).

The metastatic carcinoma of the colon in a 78-year-old man was a papillary type of neoplasm consisting of atypical acini of columnar cells supported by a loose stroma. The tumor was associated with the production of mucin. The latter gave a very intense phosphatase reaction (fig. 12).

The carcinoma of the liver in a 57-year-old man was a multicentric hepatic-cell lesion. The areas studied revealed sheets and groups of oval-shaped, anaplastic hepatic cells. Spaces between these tumor cells that corresponded to sinusoids stained intensely.

Discussion

The very sharp localizations and high chromogenicity obtained with AS-phosphates are dependent upon their basic molecular configuration and affinity for proteins. In the dyestuff industry this affinity (substantivity) in relation to textiles has been carefully studied and gives us insight into the histochemical reaction. So far as localization is concerned, the higher molecular weight naphthols generally have higher substantivity (*15*). The complex arylides as indicated in I through IV were developed by the dyestuff industry not only to give variations in shade but also, even more important, to have a greater affinity for the textile. On the other hand, the substrates derived from these naphthols were found to be readily soluble and rapidly hydrolyzed. The following compares the relative substantivity and solubility characteristics of the AS-naphthols used. Although the conditions of histochemical use differ from those

of dyeing procedures, the theoretical and practical basis for use of these naphthols as histochemical reagents is suggested.

Naphthol	*Substantivity*	*Solubility*
β-naphthol	very poor	high
α-naphthol	better than above	high
AS (unsubstituted)	10 percent	low
AS-D	12 percent	low
AS-OL	13 percent	low
AS-LT	15 percent	very low
AS-MX	17 percent	exceedingly low
AS-BS	18 percent	exceedingly low
AS-TR	20 percent	exceedingly low
AS-CL (RCA)	25 percent	exceedingly low
AS-BI	very high (over 40%)	exceedingly low

Note: This material is partly based on technical literature from the Pfister Chemical Works, Ridgefield, New Jersey.

The high substantivity of the AS-naphthols is also associated with the CONH grouping which presumably furnishes another site for hydrogen bonding. It is interesting to note that methylation of this grouping diminishes or abolishes substantivity (*16*). The great increase in chromogenicity over simple naphthols is associated with chromophore characteristic of the benzene ring as well as the bathochrome effect of the amide grouping (*17*). The presence of substituents on the benzene ring not only increases substantivity but also has an auxochrome effect, which increases chromogenicity. Electron-donating groups such as CH_3, OCH_3, and Br are potential auxochromes, since it is known that deep shades of color result if resonance increases.

Substantive naphthols are also known to exhibit a linear as contrasted with an angular structure. Decrease in this linearity profoundly affects affinity (*18*). In addition, the aromatic nuclei must assume a coplanar arrangement. Further fastness is conferred when the binding links, *e.g.*, OH and CONH, are situated alternately at a distance of approximately 10 Å or less, which corresponds to the periodicity of certain groupings in both vegetable and animal tissues (*19*). In the case of tissues, it is obvious that their complex molecular structure may make interpretation of their configuration with dye molecules difficult. Robinson (*20*) and Bamford *et al.* (*21*) studied the mechanism of dyeing the synthetic polypeptides such as polyglycine. Factors such as attachment to the side chains and backbone of the Pauling helix were considered. Three possible sites of attachment of dye molecules were polypeptide backbones, terminal amino groups of the chains, and side chain groupings. It was also pointed out that the helical configuration might be altered by the dye and concomitant hydration. In any case, it is apparent that protein chains contain many potential sites of attachment for binding of the complex naphthol AS molecules. In the final analysis, however, the sharp localization obtained with tissue sections is the criterion of usefulness.

Naphthol AS-BI phosphate (text-fig. 1) is an interesting substrate because of its brominated naphthalene nucleus. Naphthol AS-BI, which is used to produce very fast bleach-resistant dyes, combines the advantages of an AS-naphthol with the increased chromogenicity and substantivity of a brominated naphthol. Sections incubated in a solution of AS-BI show no evidence of nonspecific adsorption of naphthol. This is in sharp contrast with 6-bromo-2-naphthol which is nonspecifically adsorbed by protein (*22*). Although AS-BI phosphate is not hydrolyzed quite as rapidly as the other AS substrates, it is useful because it gives extremely sharp localizations. Of interest is the observation that fatty acid esters of AS-BI (*e.g.*, the acetate) are not appreciably hydrolyzed by tissues (*23*). This is also true of acetates of AS-CL, AS-ITR, AS-BS, and AS-TR, even though their corresponding phosphates are useful histochemical substrates. The effect of nuclear substituents upon enzyme specificity and hydrolysis rate warrants further investigations.

As previously indicated, the AS-naphthols released by enzymatic hydrolysis will couple efficiently over a wide *p*H range. It is of interest that in the dyestuff industry these compounds are coupled at or below neutrality. This is in sharp contrast with the simple unsubstituted naphthols, which couple well only in the alkaline range. In addition, the substrates presented in this paper are highly stable, even in stock solution. This also is in contrast with such substrates as 6-benzoyl-2-naphthyl phosphate, which exhibits appreciable spontaneous decomposition in solution (*24*). A *p*H of 8.0 to 9.0 was found to be optimal for the histochemical use of AS-phosphates. This is somewhat lower than the optimum described for purified phosphatase preparations. However, it has been pointed out by Neumann, that the *p*H activity-curve of phosphomono esterase is related to the substrate concentration. At a low concentration (as used in the present study), the curve shifts toward neutrality (*25*).

Recently Defendi has called attention to the ability of certain brominated and benzoylated naphthols to become adsorbed nonspecifically on tissue, and to couple to produce a dye (*26*). In practice such a situation might occur during incubation if there was some small degree of spontaneous hydrolysis. In the case of 6-benzoyl-2-naphthyl phosphate, even very small amounts of free naphthol can be concentrated by structures such as elastic tissue and thus give a spurious reaction (*27*). The use of AS-substrates, as herein described, avoids any of the aforementioned difficulties.

The reaction of developing tooth structure warrants brief comment. In the original study of phosphatase in developing teeth, Engel and Furuta described phosphatase activity of the stratum intermedium but not the ameloblastic zone (*28*). More recently Bevelander and Johnson, using the same Gomori procedure, reported activity of ameloblasts (*29*). The findings described in the present work would support the original contention of Engel and Furuta, which indicated a lack of ameloblastic activity.

Conclusions

A simple, convenient, and inexpensive procedure for the synthesis and histochemical use of several new naphthol AS substrates for the demonstration of phosphatases is presented. The method gives very sharp localizations with formalin-fixed frozen sections, as well as with acetone-fixed and frozen-dried material and blood films. This technique should be useful in laboratories of pathology since the substrate may be incorporated into a very stable stock solution which is available for immediate use.

For most purposes naphthol AS-MX, AS-TR, and AS-CL phosphates are suitable. For the most precise localizations with carefully fixed tissue (*e.g.*, frozen-dried), naphthol AS-BI phosphate is recommended.

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Naphthol AS-MX phosphate and Red Violet LB salt used unless otherwise indicated. (All photomicrographs taken at a uniform magnification of × 285. Nuclear counterstain in some preparations is hematoxylin.) Photomicrographs taken by Mr. John McGuire.

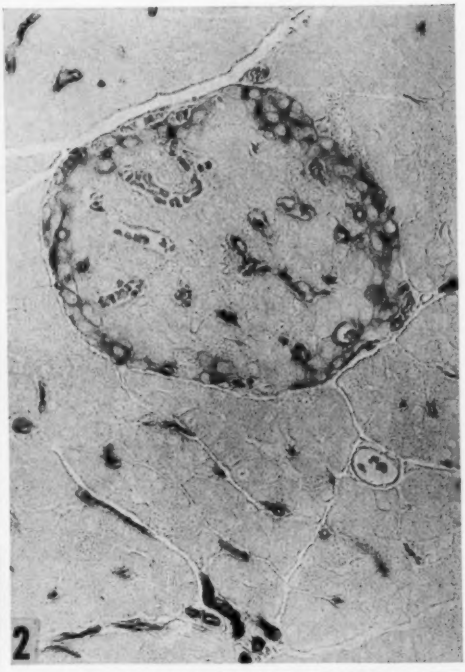
PLATE 53

FIGURE 1.—Rat kidney showing acid-phosphatase activity of both brush border and cytoplasm of proximal convoluted tubules. Four-hour incubation.

FIGURE 2.—Alkaline-phosphatase activity of pancreatic islet (rat), and adjacent capillaries. Forty-five-minute incubation.

FIGURE 3.—Alkaline phosphatase of myo-epithelium of human breast. One-hour incubation.

FIGURE 4.—Alkaline-phosphatase activity of basement membrane and associated capillaries of tongue and palate epithelium (hamster). Thirty-minute incubation.



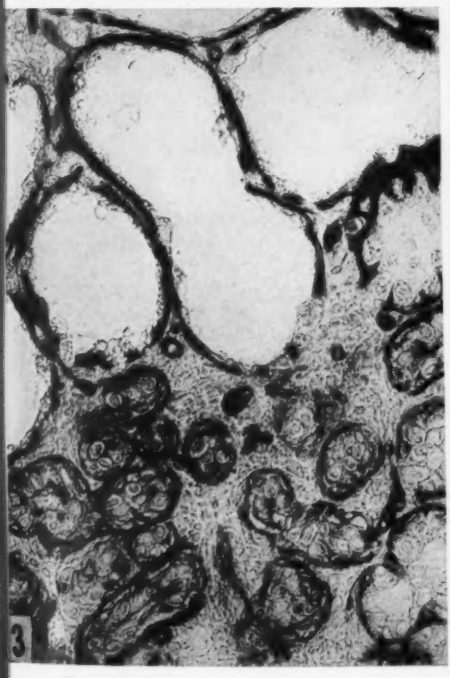


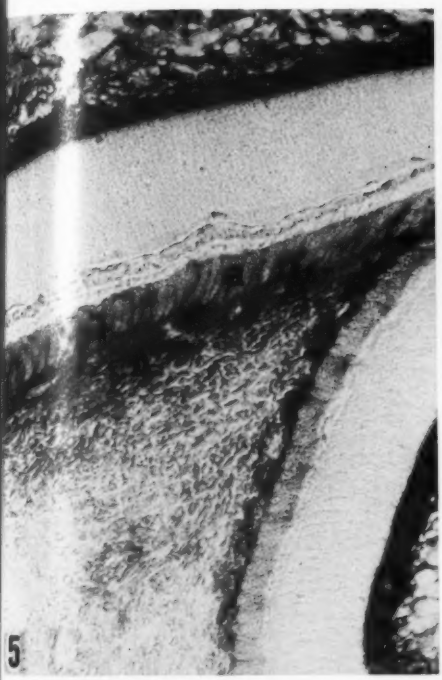
PLATE 54

FIGURE 5.—Alkaline phosphatase of developing hamster molar. Activity is present in the stratum intermedium, (*a*) odontoblasts, and (*b*) pulp. Thirty-minute incubation.

FIGURE 6.—Alkaline-phosphatase activity of granulocytes in bone marrow (hamster). Thirty-minute incubation.

FIGURE 7.—Adenocarcinoma of lung showing alkaline-phosphatase activity of border of groups of tumor cells. Forty-minute incubation.

FIGURE 8.—Carcinoma of ovary, alkaline phosphatase. Two-hour incubation, AS-BI phosphate.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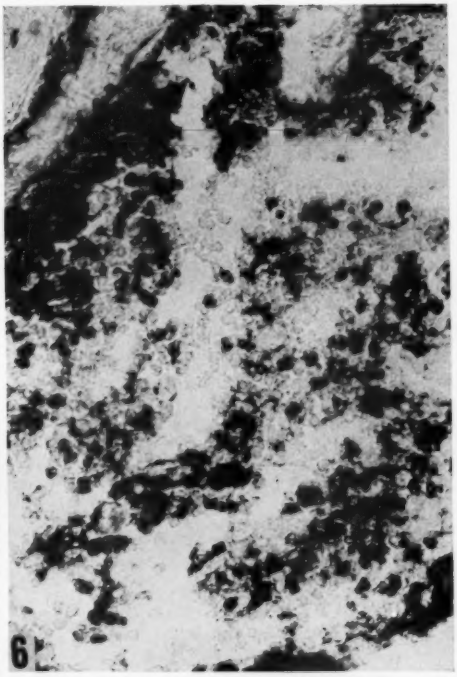
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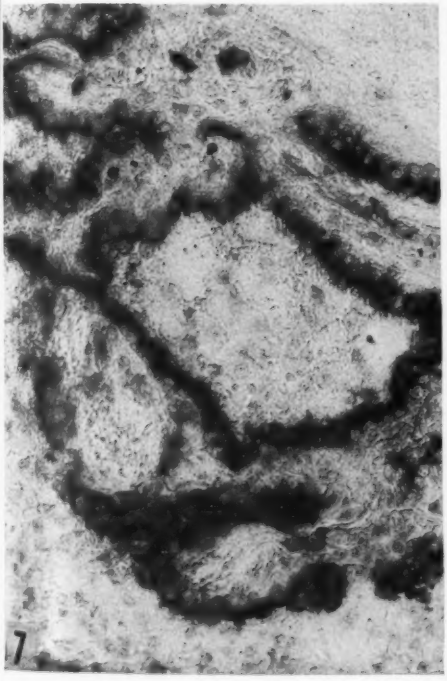
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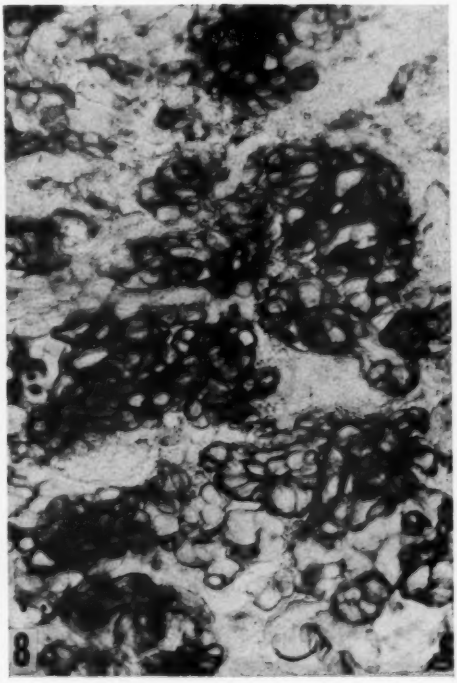
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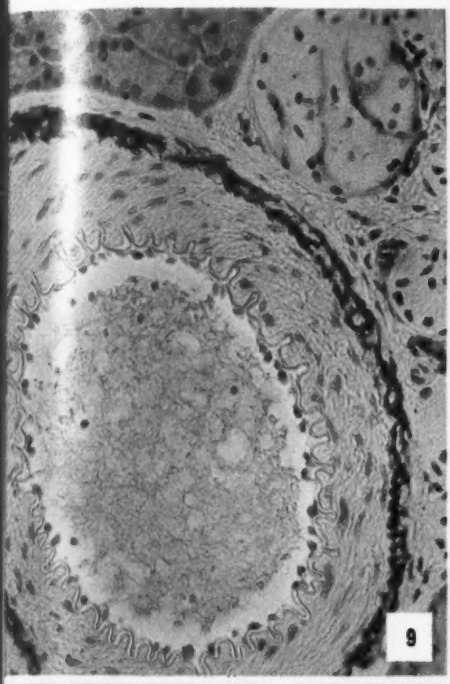
PLATE 55

FIGURE 9.—Alkaline-phosphatase activity of adventitia of arteriole. Thirty-minute incubation.

FIGURE 10.—Sharp localization of alkaline-phosphatase activity of brush border of convoluted tubules of rat kidney. Thirty-minute incubation.

FIGURE 11.—Alkaline phosphatase of mouse osteogenic sarcoma. No extracellular localization was observed. Thirty-minute incubation, AS-TR phosphate.

FIGURE 12.—Carcinoma of colon showing alkaline-phosphatase activity of abnormal mucin. Thirty-minute incubation.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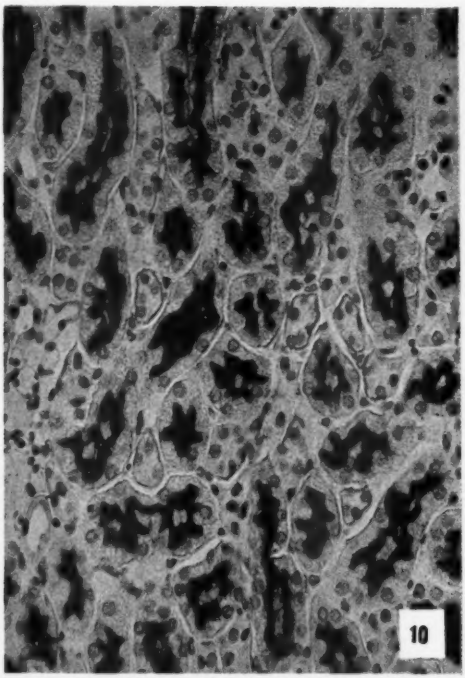
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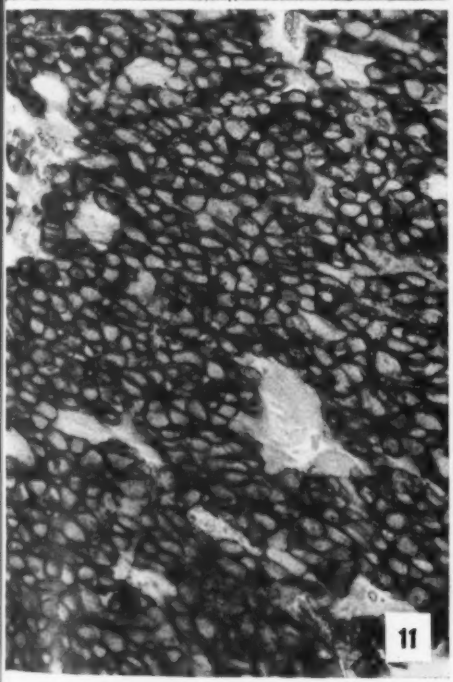
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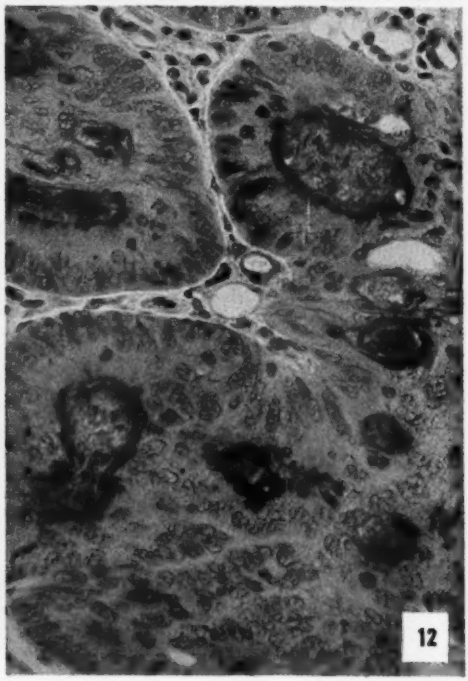
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Genetic Factors Influencing Irradiation Protection by Bone Marrow. II. The Histocompatibility-2 (*H-2*) Locus [1]

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Inoculation of homologous or heterologous [4] bone marrow protects mice against lethal, total-body X irradiation during the first 30-day post-irradiation period (*1*). When inbred mice are irradiated and treated with homologous tissue, there is an initial recovery period, but it is followed by a greatly reduced longevity compared to animals treated with isologous marrow (*2*). Following presentation of evidence that the marrow of the irradiated host is recolonized by that of the donor (*3–7*), it became evident that the genetic laws governing tissue transplantation should be tested in the case of the marrow graft in the irradiated host. It was found that in treating certain F_1 hybrids, the hybrid marrow afforded better protection than marrow of either parental strain (*8*). When marrow of either parental strain was used to protect these hybrids, death occurred during the second and third months postirradiation period.

Ordinarily, F_1 hybrid tissue is incapable of rejecting tissues from either parental strain because the histocompatibility factors of the parental strains are contained in the F_1 host. On the other hand, the parental host rejects F_1 hybrid tissues. Here parental tissue reacts immunologically against antigens of the F_1 hybrid not shared by the parental tissue. The F_1 host, however, contains some histocompatibility factors foreign to parental grafted tissue and, at least genetically, the grafted parental tissue is qualified to react against the host. Apparently this situation occurs at a time when the immunological responses of the host are inactivated by lethal irradiation.

Since susceptibility and resistance to tissue transplantation in the mouse are determined by multiple, partially dominant genes called "histocompatibility genes" (*9, 10*), the function of certain identified histocompatibility genes in protection following total-body X irradiation became the focal point of study. From this study it was shown that a single gene difference between the marrow donor and the marrow recipient produces

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[2] The authors would like to express their sincere thanks to Mr. Henry Meyer of the Radiation Branch of the National Cancer Institute for his valuable assistance in the irradiation of the animals used in this study.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[4] Isologous refers to individuals of the same inbred strain (same genotype); homologous refers to individuals of different strain but the same species (different genotype); heterologous refers to individuals of different species.

a survival pattern like the one that follows inoculation of homologous or heterologous marrow. This is an extension of work previously reported (*11*).

Materials and Methods

Coisogenic lines.—For the purpose of studying histocompatibility genes and their relation to susceptibility and resistance to tumor transplantation, Snell (*12*) has synthesized isogenic resistant lines of mice. In addition, specific mutations at histocompatibility loci have occurred in standard inbred strains of mice. These mutations have been precisely identified genetically and new lines of mice isolated. The new line is isogenic with the original strain, except for a single histocompatibility gene, and they form coisogenic pairs. It can be shown, with possible rare exceptions, that resistance to normal and to tumor grafts is a result of the single gene difference. Either member of the coisogenic pair is resistant to grafts from the other member of the pair.

One histocompatibility locus, which governs susceptibility and resistance to tumor transplantation as well as to other homografts, has been designated as *H-2* and has at least 10 known alleles. Two additional histocompatibility loci that have been characterized genetically are known as *H-1* and *H-3*. It has been predicted that from 7 to 14 histocompatibility loci are concerned with susceptibility or resistance to homografts. The present study is concerned only with the *H-2* locus.

In order to study the effect of the *H-2* locus on the successful prevention of mortality following a lethal dose of X irradiation, the C57BL/10 strain was selected. This is a standard inbred strain in which the isogenic resistant line arose by a single gene mutation at the *H-2* locus. Appropriate genetic tests have shown that the $H\text{-}2^b$ allele had mutated to the $H\text{-}2^d$ allele. C57BL/10 $H\text{-}2^b$ and C57BL/10 $H\text{-}2^d$ form coisogenic pairs. This single gene difference is sufficient to prevent transplantation of either normal or neoplastic tissue between these 2 lines (*13*, *14*). These coisogenic lines and their F_1 hybrids were used in the following experiments.

Experimental conditions.—At 3 to 4 months of age mice were exposed to 700 r X irradiation (800 r for the hybrids), which is approximately a 30-day LD95 for these coisogenic lines. Two X-ray tubes placed opposite each other were operated at 200 kvp and 15 ma.; filtration was 0.25 mm. Cu and 0.55 mm. Al with 54 cm. from each focal spot to the center of the mouse, and with a dose rate of 120 r per minute. All irradiated mice were randomized before dividing them into experimental groups. Tail-vein inoculations of marrow from 4 long bone equivalents were completed within 3 hours of irradiation, as previously described (*8*). At least 3 groups were run concurrently: the irradiated control (noninoculated) group, the irradiated group receiving isologous marrow, and a group receiving coisogenic or F_1 hybrid marrow.

Results

As shown previously (*8*), two distinct phases of the survival pattern following lethal total-body X irradiation and marrow inoculation are

recognized: 1) the acute phase, which covers the first 14 days' post-irradiation, in which the untreated control mice succumb and 2) the secondary phase,[5] which occurs only following the inoculation of certain homologous marrow cells.

Extreme amyloidosis of the kidney and a marked susceptibility to pneumonia were found in these coisogenic lines, but were less pronounced in the F_1 hybrid. In mice treated with isologous marrow, these complications account for the low survival values obtained during the 2nd and 3rd months' postirradiation and does not represent a secondary phase. These are apparently strain differences as they were not found in any other inbred strain or F_1 hybrid used in this laboratory.

Table 1 shows the survival pattern of 3 experimental groups of C57BL/10 (*H-2*^{d}) mice receiving inoculations of isologous, coisologous, or F_1 (*H2*^{b} *H2*^{d}) hybrid marrow following 700 r total-body X irradiation. In the group receiving isologous marrow there was the usual graying of the hair and a complete absence of the severe diarrhea and resulting extreme emaciation found in the "typical" syndrome of the secondary phase of the irradiation syndrome after protection with homologous marrow. Animals in which amyloidosis is found at necropsy do exhibit a resulting emaciation not typically found in irradiated animals treated with isologous marrow. The mice receiving the coisologous marrow showed an early secondary phase of an extreme type. There was severe diarrhea, extreme emaciation, dermatitis, and a lack of graying of the hair. The dermatitis probably represents a strain peculiarity since it has not been observed in other inbred strains and hybrids treated in this laboratory but has been reported by others (*15*). The animals receiving F_1 hybrid marrow showed a recovery pattern and gross appearance that could not be distinguished from the animals receiving isologous marrow. Table 2 shows the distribution of survival for a sample of the 18 experiments included in table 1.

Since mice of the *H-2*^{d} line were available in larger numbers than those of the *H-2*^{b} line, the former, C57BL/10 (*H-2*^{d}), were used as recipients in most of the experiments. Reciprocal tests were also made. Table 3

TABLE 1.—*Effect of isologous* (H-2^d), *coisologous* (H-2^b), *or* F_1 *hybrid* (H2^b H2^d) *marrow on survival of C57BL/10* (H-2^d) *mice following 700 r total-body X irradiation**

Groups†	No. mice	Donor marrow	Percent survival at days:			
			14	30	60	90
I	139	*H-2*^{d}	91	78	52	45
II	73	*H-2*^{b}	90	70	23	12
III	40	F_1 hybrid	93	93	69	69
IV	72	None	9	4‡	0. 9	0. 9

*Data pooled from 18 experiments.

†Difference between I and II = 33 ± 6.9 percent; $P = < .001$; difference between II and III = 57 ± 9.2 percent; $P = < .001$; difference between I and III = 24 ± 8.9 percent; $P = .01$.

‡ Three mice died on the 30th, 43rd, and 46th days.

[5] The "secondary phase" has been referred to by Barnes and Loutit as "homologous disease" and by Congdon, first as "secondary disease" and, later, as "delayed foreign bone marrow reaction."

TABLE 2.—*Distribution of survival for a sample of the 18 experiments pooled in table 1, C57BL/10(H-2^d) mice receiving isologous, coisologous, or F_1 hybrid marrow*

Expt. No.	Isologous (*H-2^d*) marrow				Coisologous (*H-2^b*) marrow				F_1 hybrid marrow				Control; no marrow	
	Survival at days:				Survival at days:				Survival at days:				Survival at days:	
	14	30	60	90	14	30	60	90	14	30	60	90	14	30
60	10/10	10/10	9/10	9/10	10/10	9/10	0/10	0/10					0/9	0/9
74	10/10	7/10	5/10	5/10	10/10	10/10	6/10	0/10					2/13	0/13
87	7/7	7/7	6/7	6/7	6/7	6/7	0/7	0/7					1/7	0/7
168	12/15	10/15	7/15	5/15					14/15	14/15	10/15	10/15	0/4	0/4
173	5/5	5/5	5/5	4/5					4/5	4/5	4/5	4/5	1/4	1/4
194	5/5	5/5	4/5	4/5					5/5	5/5	5/5	5/5	2/4	0/4

shows the survival pattern of C57BL/10 (*H-2^b*) mice receiving isologous or coisologous marrow following 700 r total-body X irradiation. Although the numbers are relatively small, the two phases and the gross appearance paralleled the larger experiments using C57BL/10 (*H-2^d*) mice as recipients.

The survival pattern shown in table 4 is that of three experimental groups of F_1 hybrids between these coisogenic lines, which received inoculations of hybrid marrow, or marrow from either parental strain after total-body X irradiation of 800 r. The "F_1 hybrid effect," that is, better protection by F_1 marrow than by marrow of either parental strain, was evident for this particular F_1 hybrid as well as for other F_1 hybrids in which the parental strains also had different alleles at the *H-2* locus (*8*)[6]. The previously observed difference in the ability of the 2 lines to protect through the secondary phase is also shown. An earlier onset of the

TABLE 3.—*Effect of isologous* (H-2^b) *or coisologous* (H-2^d) *marrow on survival of C57BL/10*(H-2^b) *mice following 700 r total-body X irradiation**

Groups†	No. mice	Donor marrow	Percent survival at days:			
			14	30	60	90
I	38	*H-2^b*	76	74	73	64
II	15	*H-2^d*	67	40	20	20
III	39	None	0	0	0	0

*Data pooled from 4 experiments.

†Difference between I and II at 90-day survival end point = 44 ± 15.2 percent; $P = .02$.

[6] Attention should be called to the fact that the "F_1 hybrid effect" is in no way related to heterosis as suggested by Rugh (*23*). As described here, and previously, the response specifically relates to the histocompatibility differences between marrow donor and recipient.

TABLE 4.—*Effect of isologous* (H-2^b H-2^d) *or parental-strain marrow on survival of* [(C57BL/10 (H-2^b) × *C57BL/10* (H-2^d)]F_1 *hybrids following 800 r total-body X irradiation**

Groups†	No. mice	Donor marrow	Percent survival at days:			
			14	30	60	90
I	68	F_1 hybrid	94	94	90	88
II	64	*H-2*^{b}	94	94	52	44
III	41	*H-2*^{d}	93	41	0	0
IV	62	None	0	0	0	0

*Data pooled from 14 experiments.
†Difference between I and II = 44 ± 8.2 percent; $P = .001$.

secondary phase was found to be characteristic of hybrids receiving bone marrow from *H-2*^{d} donors.

Preliminary results indicate that bone marrow from BALB/c and DBA/2, although distinct and separate inbred strains, affords as good protection as isologous marrow when the irradiated C57BL/10 (*H-2*^{d}) is the recipient. These 3 strains share the same *H-2* allele, namely *H-2*^{d}, and would therefore be antigenically compatible as far as this gene locus is concerned.

Discussion

The basic genetic principles of tissue transplantation follow simple Mendelian laws, and the same principles apply to normal as well as to neoplastic tissue. Accordingly, an F_1 hybrid produced by crossing 2 inbred strains of mice will grow tumors progressively if the tumors are indigenous to either parental strain or to the specific F_1 hybrid. This is in contrast to the lack of progressive growth of F_1 hybrid neoplastic tissue in either parental strain. The parental tissue responds to the foreign antigens in the F_1 hybrid neoplastic tissue, and homograft rejection follows.

Billingham *et al.* (*16*) postulated the possible existence of a graft against host reaction in a discussion of acquired tolerance of skin homografts. Subsequently, the work of Loutit (*17*, *18*), and others (*8*, *19–21*), has given increasing evidence that the secondary phase of the irradiation syndrome is an immunologic reaction of the graft against the host.

In the lethally irradiated mouse, it is clear that parental marrow does not afford good protection in F_1 hybrid mice. In fact, the secondary phase of the irradiation syndrome is quite apparent in this situation when compared with F_1 hybrid mice receiving isologous marrow; whereas, F_1 hybrid marrow is as effective as isologous marrow in protecting the lethally irradiated parental strains through the secondary phase. The data are in accord with expectations of a graft against host reaction when we remember that though the bone-marrow graft contains no antigens (*H-2*) that are not also present in the host, the host nevertheless contains antigens that are absent from the grafted marrow and the graft has the ability to react against these foreign antigens.

Although the *H-2* alleles are probably not the only genetic factors of importance in the protection of lethally irradiated mice, they are certainly important factors. Undoubtedly, further investigations using other inbred strains and hybrids will reveal other genetic factors. At present there are only scattered data to indicate the possible existence of these other factors. For example, in the (BALB/c × DBA/2)F_1 hybrid, which is homozygous at the *H-2* locus (*H-2*^{d}), the F_1 marrow and either parental-strain marrow afforded good protection, *i.e.*, the 90-day survival for all 3 groups was 70 percent, or better, as reported previously (*8*). However, it is to be noted that although there is good protection in each group, better protection was afforded by (BALB/c × DBA/2)F_1 marrow than marrow from either parental strain. These data indicate the existence of effective histocompatibility genes other than those at the *H-2* locus or the existence of other variables. Work is in progress to determine the effectiveness of genetically identified *H-1* and *H-3* loci in the secondary phase of the irradiation syndrome. In addition, other isogenic resistant *H-2* lines in which complicating amyloidosis and pneumonia are not prevalent are under study.

In comparing data from the reports of experiments using F_1 hybrid recipients and parental-strain donors, there is observed a marked secondary phase in most cases in which the parental strains have different alleles at the *H-2* locus. There is one notable exception. Van Bekkum (*22*) reported no reduced 90-day survival in (CBA × C57BL)F_1 hybrids treated with C57BL marrow. There is, however, a marked reduction in survival when CBA marrow is used in C57BL recipients and *vice versa*. These results indicate, therefore, that these strains are not antigenically compatible. It is suggested by van Bekkum that the F_1 hybrid is possibly more resistant than the inbred strain. This may well be a situation similar to that observed in (C57L × A)F_1 hybrids (*8*) in which the signs of the secondary phase are not so extreme as those found in other hybrids receiving parental-strain marrow. Using a different hybrid of CBA, namely (CBA × A)F_1, as a recipient and strain A marrow, Loutit (*17*) reports reduced survival, comparable to other combinations in which the parental strains differ at the *H-2* locus. This exception is not particularly unexpected, and neither reduces the importance of the role of the *H-2* locus in the secondary phase of the irradiation syndrome nor disproves the theoretical graft *versus* host reaction.

Summary

It has been shown, by employing coisogenic lines of mice differing at the histocompatibility-2 locus (*H-2*), that the survival pattern, following lethal total-body X irradiation and grafting of coisologous marrow, is similar to that obtained through the use of homologous or heterologous marrow. Thus, differences in a single histocompatibility gene (*H-2*) provide sufficient basis for the severe reaction of the secondary phase.

C57BL/10 (*H-2*^{b}), a standard inbred strain, and its isogenic resistant line C57BL/10 (*H-2*^{d}) were used in this study. Isologous and F_1 hybrid

bone marrow protected mice receiving lethal X radiation, whereas early signs of severe diarrhea, extreme emaciation, and dermatitis appeared following introduction of coisologous marrow. In F_1 hybrids (*H-2*^{b} *H-2*^{d}) between the coisogenic strains, parental bone marrow (*H-2*^{b}) or (*H-2*^{d}), produced signs of the secondary phase of the irradiation syndrome, while F_1 hybrid marrow gave excellent protection.

These data afford further evidence that an immune response of graft against host is responsible for the secondary phase of the irradiation syndrome.

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Preclusion of Secondary Phase of Irradiation Syndrome by Inoculation of Fetal Hematopoietic Tissue Following Lethal Total-Body X Irradiation [1]

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Mice exposed to a lethal dose of X radiation will survive the acute irradiation damage when they are given a postirradiation inoculation of bone marrow or spleen (*1, 2*). Inoculation of isologous marrow [4] produces a lasting recovery, while homologous marrow affords only a temporary recovery; the mice succumb during a secondary phase of the irradiation syndrome.[5] The transient nature of this recovery following inoculation of homologous marrow has been a subject of extensive investigations from which two main theories have emerged. Barnes and Loutit (*3, 4*) first observed late irradiation deaths and postulated a delayed immunological response of the host to the tissue of the foreign strain. Later, Barnes and Loutit (*5, 6*) found this theory inadequate to explain their results and postulated an immune response of the marrow graft against the irradiated host. There is increasing evidence in support of this latter theory as an explanation of the secondary phase of the irradiation syndrome (*7–10*). Makinodan and his associates (*11–13*), however, have evidence which they feel supports the former theory.

Under certain, as yet undefined, conditions, a few animals may survive the reaction, whether it is initiated by the graft or the host. The ability of a few animals to survive suggests the possibility that experimental conditions may be found which will preclude this immune response, though to date various attempts to do so have met with little success (*14*).

If there exists a graft-*versus*-host reaction when adult tissue is used, fetal hematopoietic tissue might not initiate such a reaction under the same experimental conditions owing to its lack of immunological maturity. In spite of the lack of success with fetal hematopoietic tissue as a means of precluding the immune response by other investigators (*14*), several small experiments were performed. This is a preliminary report of suc-

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[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[4] Isologous refers to individuals of the same inbred strain (same genotype); homologous refers to individuals of different strains but the same species (different genotype); heterologous refers to individuals of different species.

[5] The "secondary phase" has been referred to by Barnes and Loutit as "homologous disease" and by Congdon, first, as "secondary disease" and, later, as "delayed foreign bone marrow reaction." It occurs only following the inoculation of certain homologous marrow cells.

cessful attempts at precluding the secondary phase of the irradiation syndrome.

Materials and Methods

The (C57BL × DBA/2)F_1 hybrid was selected as a recipient for C57BL tissue, because the response of the irradiated animal following inoculation of parental marrow had been thoroughly studied and the nature of the acute and secondary phases of the irradiation syndrome was well known (*8*). The C57BL donor was selected because of the early onset and severe manifestations of the secondary phase of the syndrome. Few animals survived more than 90 days.

Experimental conditions.—At 3 to 4 months of age, mice were exposed to 800 r X irradiation, which is approximately a 30-day LD98 dose for these hybrids. Two X-ray tubes placed opposite to each other were operated at 200 kvp and 15 ma.; filtration was 0.25 mm. Cu and 0.55 mm. Al with 54 cm. from each focal spot to the center of the mouse, and with a dose rate of 120 r per minute. After irradiation, mice were randomized before dividing them into experimental groups. Marrow from the 4 long bones was prepared as previously described (*8*). A homogenate of fetal liver and spleen was obtained by repeatedly drawing the tissue through a syringe. No attempt was made to separate the liver and splenic tissue as this is difficult in the very young fetus. The hematopoietic tissue from 6 or 7 embryos was suspended in 6 cc. of Locke's solution; 5 cc. were used for the inoculations (0.5 cc. per mouse) and 1 cc. for cell counting. All inoculations of marrow and fetal hematopoietic tissue were made into the tail vein and were completed within 3 hours after irradiation. At least 3 groups were run concurrently: the irradiated control (noninoculated) group, the irradiated group receiving isologous marrow, and a group receiving irradiation and the fetal hematopoietic tissue.

Results

Table 1 presents data pooled from 20 experiments showing the distribution of survival among groups of (C57BL × DBA/2)F_1 hybrids receiving inoculations of hybrid marrow, or marrow of either of the parental C57BL or DBA/2 strains following 800 r total-body X irradiation. The 14-day and 30-day survival were approximately the same for mice receiving hybrid marrow or DBA/2 marrow. In mice receiving C57BL marrow,

TABLE 1.—*Effect of isologous or parental-strain bone marrow on survival of (C57BL × DBA/2)F_1 hybrids following 800 r total-body X irradiation**

No. mice	Donor marrow	Percent survival at days:			
		14	30	60	90
109	F_1	90	90	89	89
104	C57BL	82	68	12	11
80	DBA/2	88	81	34	34
122	None	2	1.6	1.6	0.8

*Data pooled from 20 experiments.

the early onset of the secondary phase of the irradiation syndrome at 16 to 21 days is reflected in the marked reduction in survival at 30 days compared to that at 14 days; few animals survived more than 90 days. In animals receiving DBA/2 marrow, the onset of the secondary phase was 7 to 10 days later and this is reflected in a reduced 60-day survival rate.

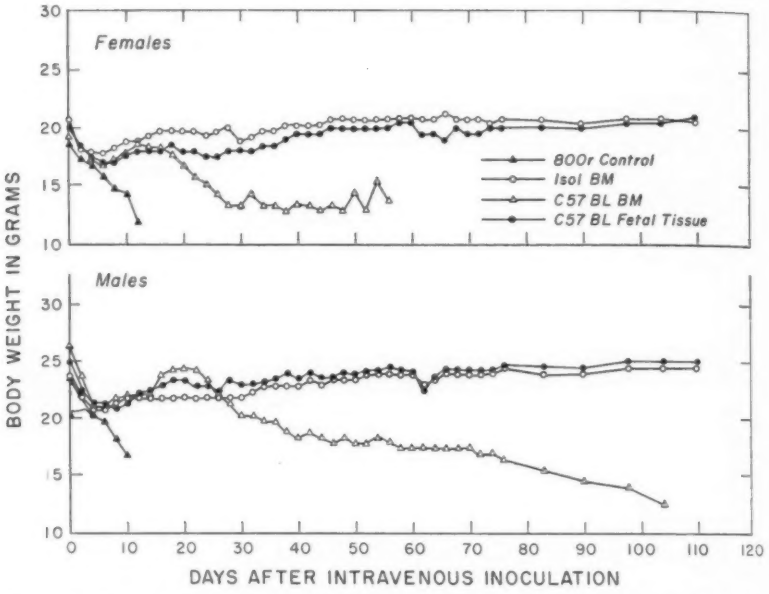
Table 2 shows the distribution of survival in one experiment consisting of 3 groups of (C57BL × DBA/2)F_1 hybrids each receiving inoculations of hybrid marrow, C57BL marrow, or C57BL fetal hematopoietic tissue following 800 r total-body X irradiation. In regard to the mice inoculated with isologous marrow, all animals that survived the acute phase of the first 14 days' postirradiation, survived 90 days. In regard to the mice inoculated with C57BL marrow, most of those that survived 14 days, survived 30 days, but only 3 males survived 90 days and all had succumbed by 108 days. These animals died with severe diarrhea and extreme emaciation; there was no graying of the hair, a "typical" secondary phase of the irradiation syndrome. The C57BL fetal hematopoietic tissue afforded excellent and lasting protection from the lethal effects of the irradiation. This group was indistinguishable from the group receiving isologous marrow. There was no evidence of a delayed reaction up to 175 days.

Text-figure 1 shows weight curves for the females and males of the 4 groups listed in table 2. To obtain an accurate record of the response in the acute phase and the brief recovery before the secondary phase, weights were recorded every 48 hours for 76 days, and weekly thereafter, until all animals receiving C57BL marrow had succumbed. The noninoculated control mice had a declining weight curve until all were dead at 12 days. The 3 inoculated groups had an initial weight loss during the first week postirradiation followed by a recovery which was permanent for the animals receiving isologous marrow or fetal hematopoietic tissue. In the group receiving C57BL marrow, there was a brief recovery period from

TABLE 2.—*Effect of isologous or C57BL marrow, or C57BL fetal hematopoietic tissue on survival of (C57BL × DBA/2)F_1 hybrids following 800 r total-body X irradiation**

No. mice	Sex	Donor tissue	Percent survival at days:			
			14	30	60	90
10	♀	F_1 marrow	90	90	90	90
10	♂		70	70	70	70
10	♀	C57BL marrow	60	60	0	0
10	♂		100	90	50	30†
10	♀	C57BL fetal liver‡	100	100	100	100
10	♂		100	100	100	100
10	♀	None	0	0	0	0
10	♂		0	0	0	0

*All groups run concurrently.
†Two died on 93rd day and 1 died on 108th day.
‡Nucleated cell count for each inoculum = 3.7×10^6 cells per 0.5 cc.



TEXT-FIGURE 1.—Weight curves for (C57BL × DBA/2)F_1 hybrid females and males showing effect of isologous marrow, C57BL marrow, or C57BL fetal hematopoietic tissue inoculation following 800 r total-body X irradiation.

the 8th to the 14th day postirradiation. The onset of the secondary phase was earlier in the females, first noted at 16 days as opposed to 21 days for the males. The gradual weight loss continued until all had succumbed.

Table 3 shows the distribution of survival among 3 groups of (C57BL × DBA/2)F_1 hybrids each receiving inoculations of hybrid marrow or fetal hematopoietic tissue from either parental strain following 800 r total-body X irradiation. The fetal hematopoietic tissue of either parental strain afforded as good protection as isologous marrow through the 90-day period, an obvious improvement over the 90-day survival produced by parental-strain marrow (table 1).

TABLE 3.—*Effect of isologous marrow or parental-strain fetal hematopoietic tissue on survival of (C57BL × DBA/2)F_1 hybrids following 800 r total-body X irradiation*

No. mice	Donor tissue	Percent survival at days:			
		14	30	60	90
8	F_1 marrow	100	100	100	100
10	C57BL fetal tissue*	100	100	100	100
10	DBA/2 fetal tissue†	100	100	90	90
8	None	25	25	25‡	12

*Nucleated cells—7.25 × 10^6.

†Nucleated cells—3.7 × 10^6.

‡One mouse died on 70th day; 1 survived. These represent 2 of the 3 control mice that did not succumb within the first 14-day period of the 122 control mice listed in table 1.

Table 4 shows the distribution of survival among groups of (C57BL × DBA/2)F_1 hybrids receiving inoculations of hybrid marrow, C57BL marrow, or C57BL fetal hematopoietic tissue following 800 r total-body X irradiation. In this experiment there was a marked secondary phase of the irradiation syndrome, with all mice showing severe diarrhea and extreme emaciation. During this period, 3 mice succumbed. The other 7 mice recovered and were indistinguishable at 90 days from the group receiving isologous marrow.[6] There are two factors that differ in the 3 experiments reported in tables 2, 3, and 4: 1) the number of cells inoculated and 2) the age of the fetal donor. The latter factor is considered the most important one. In the first 2 experiments (tables 2 and 3) the age of the fetuses was estimated to be 14 to 16 days, while in the last experiment (table 4) the fetuses were near term (19–20 days). Since the age of the fetus appears to be an important factor, experiments are in progress using fetal tissue of known age to determine the age limits within which various degrees of protection may be obtained. Other experiments testing fetal-tissue grafts in inbred strains, in which homologous marrow grafts elicit a severe reaction following inoculation into lethally irradiated mice, are in progress. Preliminary results indicate that C3Hf fetal hematopoietic tissue can produce a permanent recovery in irradiated AKR mice following 700 r total-body X irradiation, whereas C3Hf bone marrow cannot.

TABLE 4.—*Effect of isologous or C57BL marrow or C57BL fetal hematopoietic tissue (near term) on survival of (C57BL × DBA/2)F_1 hybrids following 800 r total-body X irradiation*

No. mice	Donor tissue	Percent survival at days:			
		14	30	60	90
5	F_1 marrow	100	100	100	100
10	C57BL fetal tissue*	100	100	70	70
10	C57BL marrow	90	90	10	10
5	None	0	0	0	0

*Nucleated cells—15.85 × 10^6.

Discussion

The apparent "immunological nonreactivity" of the fetal hematopoietic tissue toward the irradiated host may have its counterpart in the phenomenon of "acquired tolerance" as described by Billingham, Brent, and Medawar (*16*). They have artificially induced tolerance to skin homografts by exposing mouse embryos to living tissue antigens before the faculty of immunological response has developed. In the production of "acquired tolerance" in the embryo, the undifferentiated embryonic immune system is exposed to a fully developed antigenic system of the adult. In the experiments using fetal hematopoietic tissue to protect adult mice from the lethal effects of X irradiation, the same two systems are involved.

[6] Koller (*15*), using experimental conditions that are 100 percent lethal within 90 days, has found that when fetal hematopoietic tissue (pooled tissue from 14 to 21 day embryos) was used there was a reaction, but 7 of the 10 mice treated survived this reaction.

In one, the embryo is exposed to the antigens *in utero*, while in the other, the embryonic tissue is exposed to the antigens within the irradiated adult host. In both experiments there is apparently induced a state of "immunological nonreactivity" which is persistent. Whether the complete absence of a reaction following fetal-tissue inoculation under experimental conditions would elicit a lethal reaction if adult tissue were used, which can be explained on the basis of "acquired tolerance," or some other as yet unknown immunological phenomenon, awaits further investigation.

The partial tolerance to skin homografts induced during late fetal life appears to correspond to the response found in the irradiated host treated with tissue from a fully developed embryo. The results presented in table 4 would indicate that late fetal life is a critical period in which only partial tolerance can be induced, because there is a reaction from which some animals recover. Confirmation of the effect of the age of the embryo awaits further investigation.

There are few reports on the use of fetal homologous hematopoietic tissue in the literature. Congdon (*14*) states ". . . the fetal homologous blood-forming tissue, however, may cause less delayed reaction than adult homologous tissue." In a later report (*17*) he states that ". . . fetal liver gave better long-term survival than any other modification of the foreign bone marrow experiments." This statement is accompanied by a mortality curve for 83 (C57L $\times$ A)F_1 hybrids exposed to 900 r and treated with fetal liver. This curve shows a mortality at 90 days of approximately 45 percent with fetal tissue as opposed to approximately 85 percent mortality for 21 mice treated with homologous marrow. Since experimental details were not given, it is difficult to evaluate a higher mortality following inoculation of fetal tissues reported by Congdon.

The use of fetal hematopoietic tissue as a protective agent has been reported by Jacobson *et al.* (*18, 19*) and Russell *et al.* (*20*). These reports either are clearly not comparable with the data presented herein or are difficult to evaluate because of a lack of pertinent information. In Jacobson's experiments (*18*) CF-1 mice were used as recipients and donors. The amount of homogeneity found in the CF-1 mice at the time these experiments were conducted would be difficult to estimate because it is not a standard inbred strain. Jacobson found the embryonic liver cells to be more effective than adult marrow in increasing the 30-day survival value, but no data was presented on the period after 30 days. Later, Jacobson (*19*) used mouse embryonic liver as a heterotransplant in rabbits exposed to 900 r X irradiation (the 30-day LD50 is about 800 r). No late deaths occurred in the surviving rabbits treated with embryonic mouse liver. It is not known whether adult mouse marrow would increase the 30-day survival in rabbits exposed to sublethal irradiation or whether the adult mouse tissue would elicit an immune response in rabbits under these experimental conditions. Russell (*20*) used isologous and homologous embryonic liver following the sublethal dose of only 200 r X irradiation, in an attempt to alter the phenotype of genetically anemic mice. With this low dose of radiation, only the isologous embryonic liver inoculation

proved a successful means of permanently altering the phenotype of these anemic mice. There was no lasting alteration of the phenotype when homologous tissue was used. These data are in agreement with expected results if adult marrow were used following 200 r X irradiation (*21*). In neither case would there be an immunological response like that observed in the homologous marrow inoculation following lethal irradiation.

The data presented here on the use of fetal hematopoietic tissue are in agreement with the graft-*versus*-host reaction theory as an explanation of the secondary phase of the irradiation syndrome.

Summary

The secondary phase of the irradiation syndrome has been successfully precluded by the use of fetal hematopoietic tissue inoculation following lethal total-body X irradiation. No reaction was observed following inoculation of fetal tissue under experimental conditions that would elicit a lethal reaction if adult tissue (bone marrow) were used. The survival data are supplemented by weight curves, clearly showing the weight loss characteristically found when incompatible marrow is used and the absence of a corresponding weight loss with isologous marrow or fetal hematopoietic tissue. These data are discussed in relation to "immunological nonreactivity" and are in agreement with the theory of the graft-*versus*-host reaction as an explanation of the secondary phase of the irradiation syndrome.

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A Preliminary Report of Electron Microscopic and Bioassay Studies on the Rous Sarcoma I Virus [1]

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Since the demonstration that the Rous sarcoma was due to a filterable agent [Rous (*1*)], many investigators have attempted to characterize this agent in various essential ways but only recently have efforts been directed toward actual visualization. In the long and often disappointing course of these studies it became clear that the characterization would not be a simple one. The various approaches to the problem, that is, studies on its biological, chemical, and physical properties, were complicated by the fact that no really purified preparation of the agent was available. Consequently, results in these areas from different laboratories frequently did not agree (*2*). Only since the accomplishments of Bryan and co-workers (*3*, *4*, *5*), who made available a highly potent preparation with some increase in purity, have these studies, as well as attempts at visualization and identification, been made with some assurance of success.

Previous to this, the position of Beard (*6*), that the Rous agent had not been purified to the point where studies on its properties could have any significance, was certainly a tenable one.

Failures or, at most, minor successes were to continue in these areas, but in 1947 Claude, Porter, and Pickles (*7*) described, in spread cells of tissue cultures of Rous chicken sarcoma examined with the electron microscope, particles about 75 mμ in diameter. In this paper the ratio of cells containing particles and the number of particles per cell was not mentioned, though from the figures it could be assumed that a fair number of cells were infected. While this study was basically morphological in character and not related to bioassays of agent potency, it did raise the hope in some quarters that a clear relationship between malignancy and a definite viral particle might well be demonstrated. The problem, however, was a peculiarly complex one. For example, Bernhard and Oberling (*8*) emphasized the point that after 5 years of study they

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[5] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

were unable to find particles in the several thousands of cells examined. Shortly afterward, however, Bernhard *et al.* (*9*) reported the finding of particles in Rous sarcoma tissue cultures that had been either exposed to X rays or grown in media containing C^{14}. They pointed out the extreme rarity with which particles were demonstrable even under these most favorable conditions. Here again no attempt at correlation with bioassay determinations of potency was made.

Gaylord (*10*) was the first to attempt the demonstration of the agent by the method of thin sectioning and electron microscopy. He carefully analyzed 3 tumors and found relatively large numbers of particles associated with the cells of the youngest tumor, a moderate number of particles in an older tumor, and no particles in the oldest; then he inferred a relationship between the number of particles and tumor age. Once more the lack of bioassay control was significant in the interpretation of results.

More recently, Epstein (*11*), in spread cells of 5 Rous sarcoma tumors grown in the ascitic form, succeeded in demonstrating a relationship between the number of cells containing particles in any given tumor and the relative concentration of agent in that tumor as determined by bioassay. Under the conditions of Epstein's experiments, he found that with optimal conditions only 1 cell in 50 contained virus particles. His results coincided with those of Carr (*12*) and Rubin (*13*) and for this reason he suggested that the Rous agent undergoes a life cycle during part of which it is not recognizable by methods now available.

Again Bernhard *et al.* (*14*) examined a series of tumors derived from both cell and extract inoculation. In this extensive study, in which 75 sarcomas were examined, no evidence was found of correlation between the number of particles and growth rate, though it was noted that tumors produced by the inoculation of a low infective dose of extract contained few particles. A constant relationship between the number of identifiable particles and the degree of infectivity of tumor extract was not found. In this case, the principal cause of lack of parallelism was laid to the difficulties inherent in the technical methods used. The authors also felt that the existence of a life cycle during part of which the virus might not possess the same corpuscular aspect could explain the variation in number and relative scarcity of particles.

At the inception of this study it was proposed to demonstrate by a combination of bioassay and particle counts of thin sections of tumor, whether or not the primary factor, which needs to be considered in relating viral particles of the Rous tumor to the potency of extract of that tumor on reinoculation, is that of concentration of particles. The observations that follow constitute the results of a preliminary study in which two groups of tumors were analyzed, one group induced with a high concentration of agent, the other with low. A more detailed study involving a larger and more closely graded series of tumors is at present under way and will be reported at a later date.

Materials and Methods

Samples of tumor tissue were obtained from tumors induced by inoculation of cell-free extracts of known potency. Portions of the tumors from which the samples were taken were then used to prepare extracts for bioassay. Tissue was taken from one or more areas of each tumor and fixed for 1 hour in chrome-osmium (*15*) and/or veronal-acetate-buffered 1 percent osmium tetroxide at *p*H 7.2 (*16*). After fixation in chrome-osmium, the tissues were washed in 0.85 percent NaCl to remove excess chrome solution, dehydrated through a graded series of alcohols, and embedded in a mixture of 1 part methyl methacrylate and 5 parts *n*-butyl methacrylate. Prepolymerization of the embedding matrix was regularly performed. Sections were cut on a Porter-Blum microtome (*17*) set to cut at 250 A°. Representative sections were obtained from at least 2 tissue blocks of each tumor, and a minimum of 200 different cell sections were examined. Only sections of cells cut through a portion of the nucleus were considered. The number of cell sections containing particles, as well as the number of particles per cell section, was determined. Particles were counted if they were clearly contained within the cytoplasm or if they were in direct contact with, and apparently adherent to, the plasma membrane.

Pellets obtained by sedimenting partially purified virus suspension were fixed for 1 hour in citrate buffer *p*H 7.2 containing 1 percent osmic acid. Dehydration, embedding, and sectioning were carried out in the same manner as for samples of tumor tissue.

Electron micrographs were taken with an RCA model EMU 2C and a Siemens Elmiskop I electron microscope.

Results

The fine structure of Rous sarcoma I cells has been adequately described by Gaylord (*10*), Bernhard, Oberling, and Vigier (*14*), and later by Epstein (*18*), and will be briefly summarized here. The nuclei of these cells are large in relation to the size of the cell body and the nucleoli frequently hypertrophied. In many of the cells of tumors induced with high doses of extract, spaces within the ergastoplasmic membranes are characteristically distended and contain a fairly dense precipitate (fig. 1). Mitochondria regularly show blebbing and distortion of their internal membranes (fig. 1). These modifications were not found as consistently in cells of tumors induced with low doses of agent.

Examination of cells from the series of tumors induced with high doses of agent showed the presence of particles of the characteristic size (75 mμ) and shape first reported by Gaylord (*10*), and in excellent detail by Bernhard *et al.* (*14*). The particles are slightly ovoid and possess outer and inner membranes surrounding a central electron-dense core. Our measurements of the components of the particles correspond to those of Bernhard, Oberling, and Vigier (*14*), the total diameter of the particle averaging 750 A° and the dense inner core averaging 350 A° (fig. 2).

The particles are located in characteristic situations among the tumor cells. They are found essentially at two sites: 1) along the outer surface of the plasma membranes to which they often seem to adhere—their arrangement along this surface can be extraordinarily regular; 2) inside intracytoplasmic vacuoles, the origin of which is not always clear. They might be enlarged Golgi vacuoles or swollen mitochondria and often appear as the result of an invagination of the surface membrane later secluded from it (fig. 1, arrow).

In our material, areas suggestive of the formation of viral particles from intracytoplasmic "viroplasm," as described by Bernhard, Oberling, and Vigier (*14*), have not been detected up to now. Rather, the impression was gained that the concentration of particles is primarily extracellular and that the majority of intracellular particles are in this position as the result of incorporation through phagocytosis, because no particles were found free in the cytoplasm and not surrounded by a membrane.

The amount of particles differs in a striking manner in tumors induced with high *versus* low infectious doses.

Table 1 shows the results of counts made of these particles in which their number per cell section and the percentage of cells containing them was determined. It should again be emphasized that cells were considered as possessing particles if the particles were either clearly contained within the cytoplasm or in direct contact with, and apparently attached to, the plasma membrane (fig. 1). In the high-dose group, tumors 1, 5, 6, and 7 were induced by an extract of a "high-dose" tumor, which was related to the standard virus preparation CT 629 by bioassay (*4*). The other tumors in this group, 2, 3, 4, 8, 9, and 10, were induced by the inoculation of the undiluted standard CT 629. The low-dose tumors, 11, 12, and 13, were induced by a 10^{-7} dilution of standard preparation CT 629 as indicated in column 1 of table 1.

Considering only positive cells and cells of tumors induced with high dose of agent, the number of particles per cell section varied from 1.6 to 5.9. When all cells counted are included, the number varied from 0.29 to 5.58. The percentage of cell sections possessing particles varied from 16 to 94.5. The values obtained indicating the average number of particles per cell section appear to be low but it should be remembered that they represent counts made on single sections approximately 30 mμ thick. If Rous sarcoma cells are conservatively estimated to be 10 μ in diameter, over 300 serial sections could be cut through one cell and thus the number of particles *per cell* would be at least 200 times greater than the values obtained by this method. It should be noted further that, within this group of tumors induced with a high dose of extract, there is a rough correlation between the percentage of cell sections with particles and 1) the number of particles per cell section, and 2) the concentration of agent recovered from these tumors on bioassay. The difference between the high- and low-dose tumors in regard to the number of particles present is obvious. Only 3 of the low-dose tumors examined could be included in the table and, in addition, a separate group of 4 tumors selected for

TABLE 1.—*Summary of data on individual tumors; arranged in order of the amount of virus in their extracts as determined by bioassay*

Tumor identification				Determinations on tumor												
Group	Initiating dose * of virus	Host chicken (*No.*†)	Tumor designation (*Serial No.*)	Latent period (*days*)‡	Time of growth in host (*days*)		Virus extracted (*Log ED50 per gm.*) §	Incidence of cell sections containing particles			Average number of particles per cell section					
					From virus inoculation	From macroscopic appearance		Fraction		Mean (*per-cent*)	Positive sections only			All sections		
								Sample a.	Sample b.		Sample a.	Sample b.	Mean	Sample a.	Sample b.	Mean
"High-dose"	10^{-2}	881	1	5	7	2	8.6	90/100	75/100	82.5	6.10	4.04	5.07	5.49	3.03	4.26
	10^{0}	778(L)	2	5	8	3	8.5	69/100	72/100	70.5	2.01	1.83	1.92	1.39	1.32	1.36
	10^{0}	777(R)	3	5	8	3	8.3	73/100	79/100	76.0	4.01	3.52	3.77	2.93	2.68	2.81
	10^{0}	776(R)	4	5	8	3	8.2	94/100	95/100	94.5	4.34	7.48	5.91	4.08	7.07	5.58
	10^{-2}	715	5	5	7	2	8.1	56/100	49/100	52.5	4.36	5.61	4.99	2.44	2.75	2.60
	10^{-2}	823	6	5	7	2	8.0	56/100	38/100	47.0	2.55	4.32	3.44	1.43	1.64	1.54
	10^{-2}	103	7	5	8	3	8.0	67/100	70/100	68.5	3.66	4.54	4.10	2.45	3.18	2.82
	10^{0}	776(L)	8	5	8	3	7.7	39/100	74/100	56.5	2.36	3.86	3.11	0.93	2.86	1.90
	10^{0}	777(L)	9	5	8	3	7.3	10/100	22/100	16.0	1.20	2.05	1.63	0.12	0.45	0.29
	10^{0}	778(R)	10	5	8	3	7.3	47/100	29/100	38.0	3.08	1.59	2.34	1.31	0.29	0.80
"Low-dose"	10^{-7}	675	11	12	44	32	2.9	0/100	0/100	0	0	0	0	0	0	0
	10^{-7}	917	12	16	26	10	2.8	0/100	0/100	0	0	0	0	0	0	0
	10^{-7}	541	13	14	26	12	2.7	0/106	2/111	0.9	0	1.00	0.5	0	0.046	0.023

*Amount of virus inoculated in terms of the equivalent dilution of a standard virus preparation (CT 629); all inocula were of 0.2 ml. volume.

†Leg band number of host chicken. Numbers followed by (L) or (R) indicate tumors in left and right wings, respectively; other numbers represent chickens inoculated in the left wing only.

‡Time from inoculation of virus to macroscopic detection of a tumor.

§Each tumor extract was assayed in relation to standard virus. The log potency ratios within assays were added to the average value of Log ED50 for the standard over-all assays. The log quantity of virus is computed per gram of wet weight of tumor.

low concentration of agent by bioassay but carried by trocar transplant *(19)*, were also analyzed. In 1 of these, 4 particles were identified on examination of 230 cell sections and in the 3 others no particles were found in any of the 600 cell sections examined.

It was considered important to examine samples of pellets obtained by sedimenting partially purified virus suspensions derived from tumors induced with high and low dose of virus. Figure 3 is a representative electron micrograph of a thin section of a pellet obtained from a "low-dose" tumor. Particles bearing an identical morphology to those found in the whole tissue are seen but in small number in relation to the amount of background material. Figure 4, on the other hand, shows a pellet obtained from a "high-dose" tumor and illustrates an area where there are numerous particles in a high concentration. In addition to the many clearly recognizable particles, the short strands and filaments, with an electron density like that of the particles, are thought to represent particles damaged in the process of preparation of the pellet.

Discussion

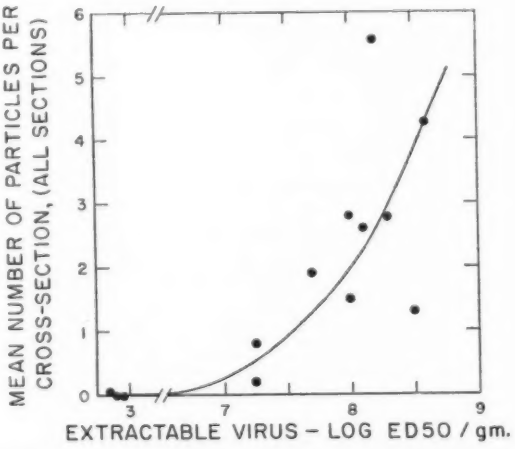
In order to relate, beyond any reasonable doubt, a causal agent with a disease entity, certain definite requisites must be established. The most important of these is the demonstration of a consistent and quantitative relationship between the severity of the disease and the number of the presumed causal agents present. Secondarily, the disease must have consistent features by which it may be identified and this also applies to the causal agent itself. Particularly in the Rous sarcoma, one could consider the sarcoma cell and its capacity to proliferate as the disease entity and the viral particles frequently shown to be associated with it as the causal agent.

In the efforts made to date in this direction, one or more of the essential requisites have been absent. To make the relationship clear, the study must be carried out using a *cell-free* extract of *known potency* as determined by bioassay. The extract should be used as inocula into a series of animals in serial dilution or at least in high and low concentration. Following this, the infectivity of extracts from the tumor so produced must also be determined. When these biological facts are known, particle counts made on cells from such tumors would have real significance should a correlation be found between the number of cells containing particles and the number of particles per cell, and the previously determined potency of these tumor extracts. The work of Epstein, and Bernhard, Oberling, and Vigier were steps in the right direction but under the circumstances of the experiments could not be expected to provide final proof. In the present report it is felt that such proof has been provided in that statistically significant differences in the number of cells containing particles and the number of particles per cell has been demonstrated between tumors induced with high and low doses of virus. The lack of a close correlation between the number of particles and the potency of extract as determined by bioassay in the "high-dose" group

may be readily explained by the well-known variation in chicken sensitivity to infection with the Rous agent.

These results further indicate that in the present condition at least, no recourse to the hypothesis of "masked" virus (*20*) nor to the existence of a life cycle, during the ***major*** part of which the agent is unidentifiable, is necessary. The fact that in thin sections of tumor cells, from growths induced with highly potent preparations, as many as 70 to 95 percent were found to be associated with particles suggests the probability that if whole cells were examined, practically all of the cells would have been demonstrated to possess particles. It is felt that in these the constant presence of the agent assumed to be the inciting factor for induction of malignancy is directly related to the high concentration of agent in the original inocula. The good correlation between the potency of extracts from tumors induced with high and low doses of extract and the concentration of the particles present in the corresponding pellets lends strength to the supposition that the particles are indeed the causal agent. By making this assertion it should be obvious that there is no intent to imply that a life cycle does not exist or that it will not be made evident. These results concern only, for the time being, the two extreme lots of tumors obtained at the opposite ends of a progressive serial dilution experiment. They correspond to the extremities of the curve represented in text-figure 1. Statistical analysis indicates that in these two widely separate groups, the number of tumors examined provides sufficient information. The probability that these results would have been obtained by chance is less than 0.01.

It is felt, therefore, that the results of this first study indicate that the particles found in the tumors either represent the causal agent or, if not the agent *per se*, they are quantitatively related to it.



TEXT-FIGURE 1.—Graph showing the relationship between the mean number of particles per cell section and extractable virus.

Summary

Thin sections of samples of tumors induced with high and low doses of Rous sarcoma virus were examined with the electron microscope. Counts were made of the number of cell sections showing association with particles and the number of particles per cell section. The results of the counts were compared with the potency of extracts of these tumors as determined by bioassay. Both the number of particles and the potency of extracts were found to be in a much higher category in the "high-dose" group. Within this group there was also a rough correlation between the number of particles and the potency of extract. These results indicate that these particles, with their characteristic size and morphology, represent, or are quantitatively related to, the agent responsible for the induction of the Rous chicken sarcoma.

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PLATE 56

FIGURE 1.—An electron micrograph of a portion of the cytoplasm of a Rous sarcoma cell from a high-dose tumor showing, at the *upper right*, dilation of the spaces between the membranes of the ergastoplasm by a relatively electron-dense material. Viral particles are present within a vacuole at the *upper center* and continuity between the limiting membrane of the vacuole and the plasma membrane is indicated at the *arrow*. At the *extreme right*, viral particles are visible in contact with the plasma membrane. One of the mitochondria in the field shows blebbing. Chrome-osmic fixation. Approximately × 40,000

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PLATE 57

FIGURE 2.—An electron micrograph showing detail of viral particles within a vacuole. The long mitochondrion to the *left* of the vacuole shows blebbing. Chrome-osmic fixation. Approximately × 95,000

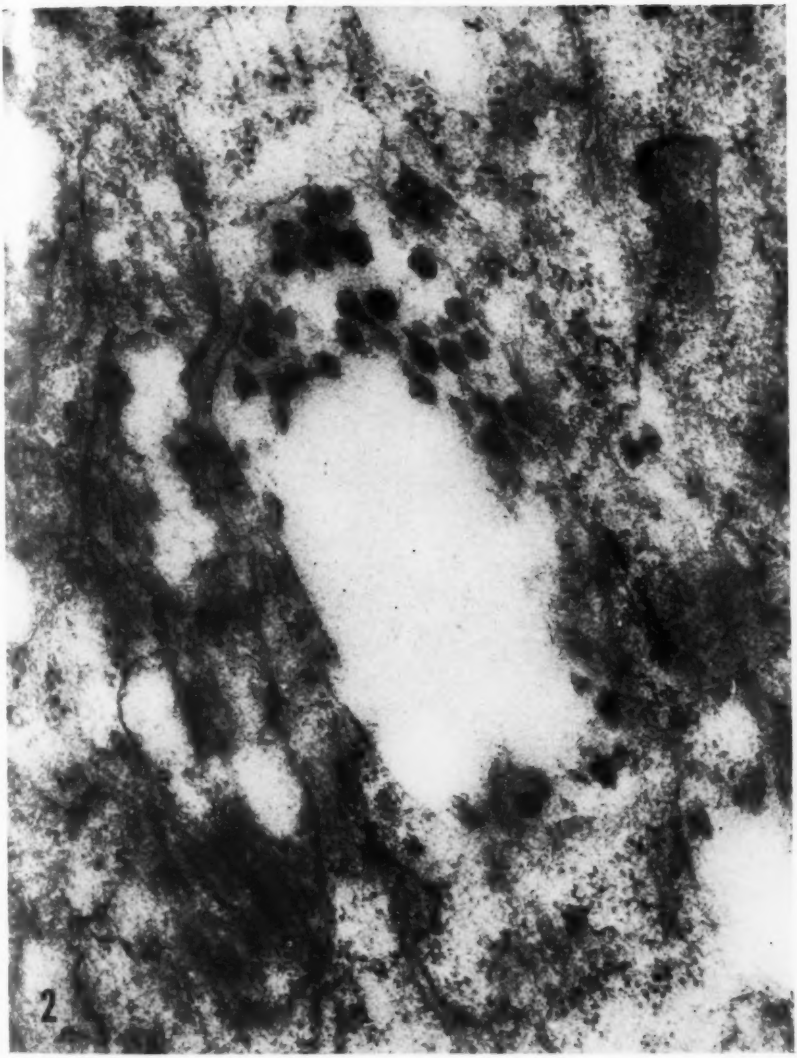
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PLATE 58

FIGURE 3.—An electron micrograph of a thin section through a pellet obtained by centrifuging an extract from a low-potency tumor. The few viral particles present in the field are circled for emphasis. Fixed in isotonic 1 percent osmium tetroxide. Approximately × 41,000

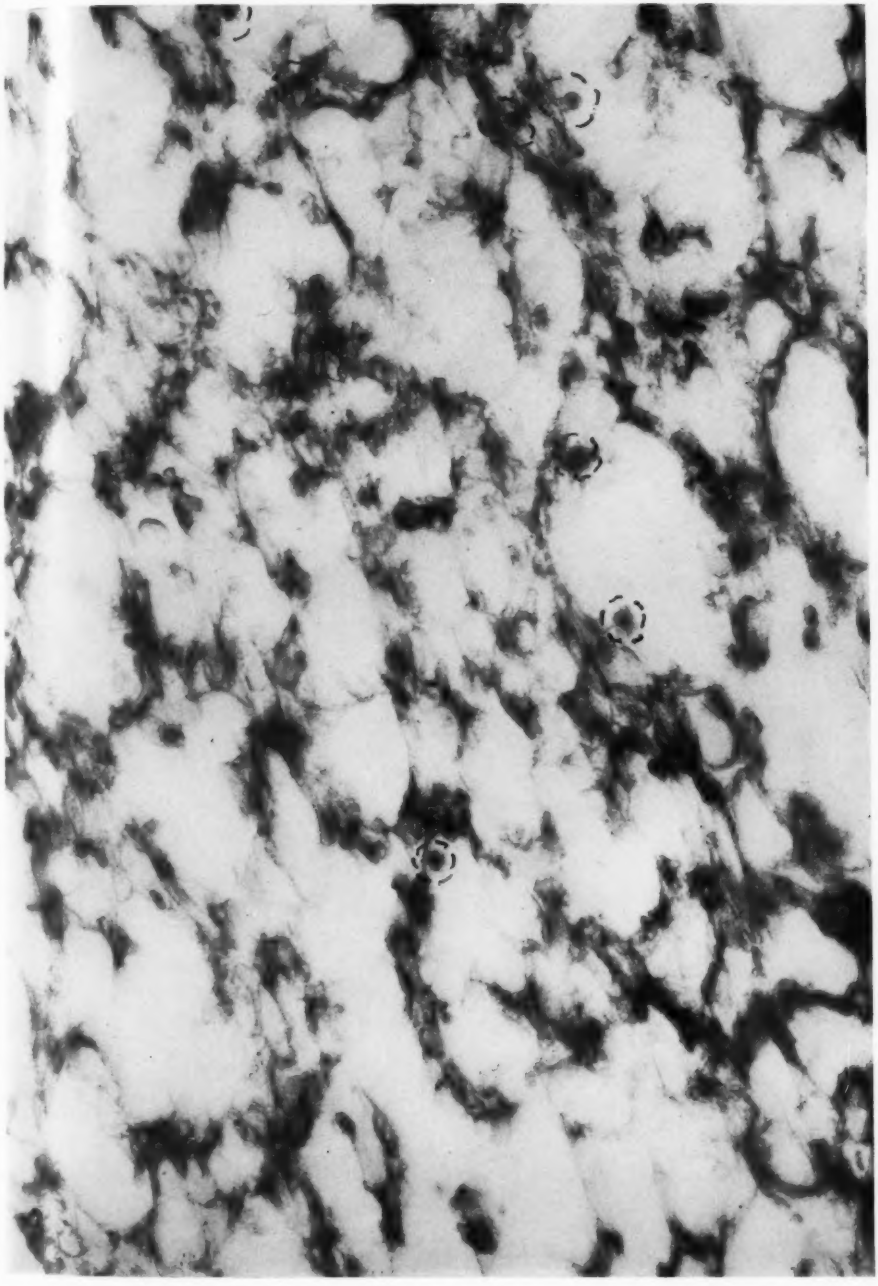
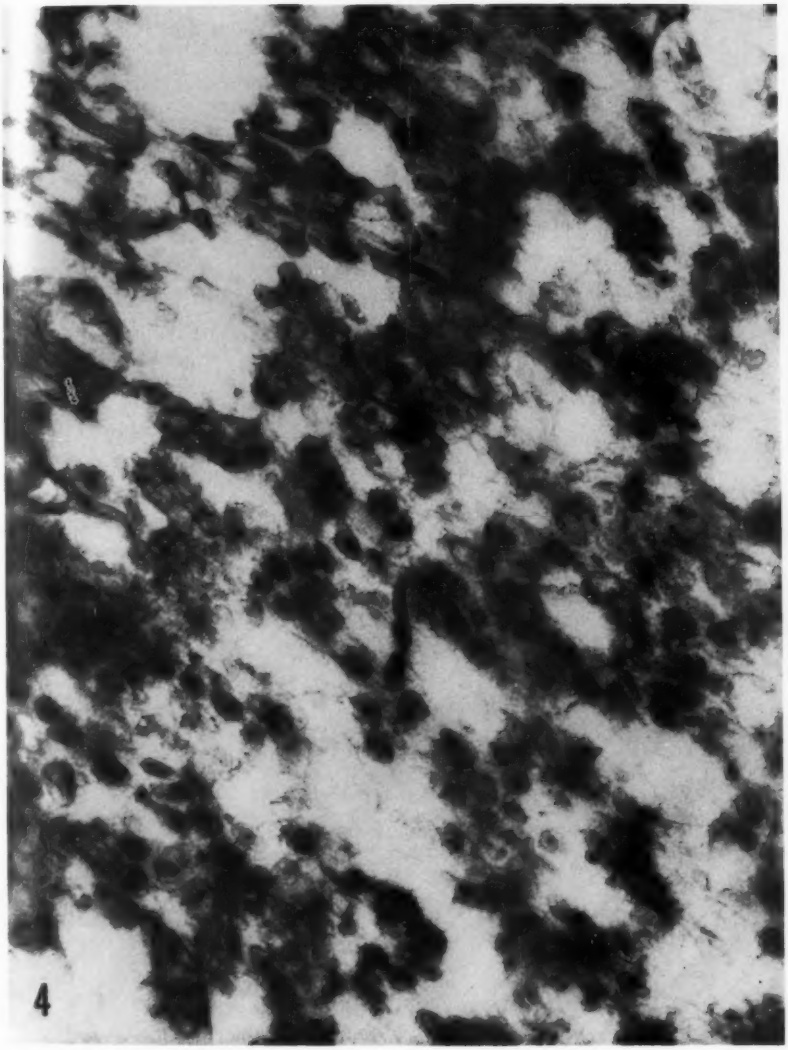


PLATE 59

FIGURE 4.—An electron micrograph of a thin section through a pellet obtained by centrifuging an extract from a high-potency tumor. Numerous viral particles with typical morphology are scattered throughout the field. Fixed in isotonic 1 percent osmium tetroxide. Approximately × 54,000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Announcement

WORLD CONGRESS OF GASTROENTEROLOGY

Host Organization: American Gastroenterological Association

The World Congress of Gastroenterology will meet at the Sheraton-Park Hotel in Washington, D. C., from May 25 to May 29, 1958. That meeting will be followed by the annual Scientific Meeting of the host organization, from May 30 to May 31, 1958, at the same hotel. An extensive program will be presented by participants from more than 40 foreign countries and from the United States. A program on Gastric Cancer to be presented during the morning session on May 29 may be of special interest to readers of this Journal. Other sessions will cover a wide range of topics. For further information, write to the Secretary-General, Dr. H. Marvin Pollard, University Hospital, Ann Arbor, Michigan.

The Journal of the National Cancer Institute is published monthly by the National Cancer Institute of the National Institutes of Health, Bethesda, Md. Twelve issues are published each year in two volumes, beginning with number 1 in July and January. The Journal publishes original observations and investigations in laboratory and clinical research in cancer. Manuscripts are considered for publication with the understanding that they have not been published previously.

Publication is not limited to members of the National Cancer Institute or to those whose work is supported by Government funds. Manuscripts should be submitted to the Journal of the National Cancer Institute, National Cancer Institute, Bethesda 14, Md.

Suggestions to Authors

1. Submit an original and two carbon copies of the manuscript. Follow the style of a recent copy of the Journal, especially for illustrations, references, and tables. The latest edition of Webster's International Dictionary is followed in matters of spelling, capitalization, punctuation, compounds, and hyphenation. Current medical dictionaries and Chemical Abstracts are usually followed for medical and chemical terms. Type material double spaced.

2. In the by-line, include the name of the institution with which each author is connected. Official title or academic degree is optional.

3. Begin each page with a new paragraph; *i.e.*, each page should contain one or more complete paragraphs, and no paragraph should carry over from one page to the next unless it is longer than a full page. Do not begin a new paragraph on a page carrying a run-over paragraph. This is a requirement of the Government Printing Office.

4. Insert each footnote in the text immediately after the line in which it is mentioned.

5. Type each table on a separate page, to be the page immediately following the paragraph in which the table is first mentioned.

6. Graphs, charts, and drawings are printed in the text and referred to as text-figures. Legends for text-figures should be included in the text immediately following the paragraph in which the text-figure is first mentioned. A drawing three times the size of that desired for the final figure is preferable. The lettering should be uniform and of such size that in the reproduction it will be approximately the same size as that of the printed type used in the text. The original drawings reproduce best and are therefore preferable. If two additional copies (photostats, etc.) of each drawing are also submitted, review of the manuscript is expedited.

7. Photographs are printed together at the end of each article, as plate pages. The maximum size for each printed plate page is 5¼ by 8 inches, and the photographs should be combined into as few plates as possible. Photographs should be submitted either in the exact size, or exactly double the size, of the final printed reproduction. Mount the photographs for each plate together on one piece of heavy cardboard. Number each photograph consecutively. Type the legends for each plate on a separate page. If, in addition, two unmounted duplicate prints of each photograph are also submitted, review of the manuscript will be expedited. Color transparencies should be accompanied by a set of black-and-white photographs.

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